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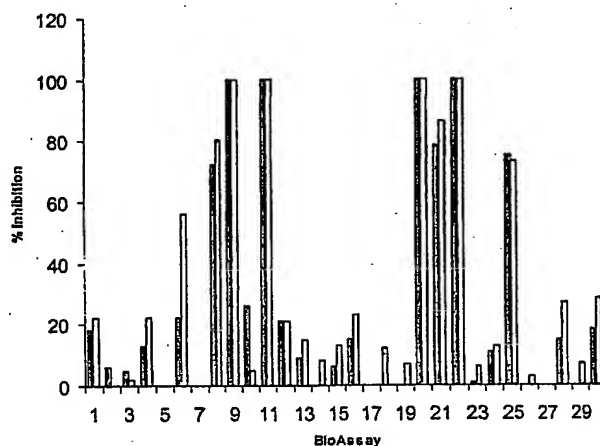
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(54) Title: STANDARDIZED FUNCTIONAL FOOD PRODUCTS



(57) Abstract: The methods of the present invention provides a standardized functional food product associated with desired health benefits. One or more extracts of an unprocessed functional food source, such as soy beans, are assayed in one or more bioassays associated with a desired physiological response or health benefit to detect one or more bioactivities of the total extract. Known or suspected chemical constituents of the unprocessed functional food source, known or suspected to be associated or correlated with the desired physiological response or health benefit, may also be assayed to determine if the bioactivity (or bioactivities) of the extract may be due to these known constituents. The extract(s) may also be fractionated to detect active components that may be responsible for the activity of the extract. The amounts and/or the bioactivities of the active components in the extract are then determined. The determination of the amounts and/or the bioactivities of the active components, as well as the total extract bioactivities, allows the development of a functional food product standard associated with a desired physiological response or health benefit against which other preparations of the same functional food product can be tested.

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## STANDARDIZED FUNCTIONAL FOOD PRODUCTS

### FIELD OF THE INVENTION

The present invention relates generally to the field of functional food products. More particularly, the present invention relates to methods for standardizing botanical extracts to create standardized functional food products or standardized dietary supplements.

### BACKGROUND

Functional foods are considered to be foods that contain significant levels of biologically active components that impart health benefits or desirable physiological effects beyond basic nutrition. For example, wheat bran, widely known as a source of dietary insoluble fiber, may reduce the risk of breast or colon cancer. Gelatin is thought to help improve symptoms associated with osteoarthritis, and cranberries may improve urinary tract health. Functional foods may be consumed in a variety of forms, or may be mixed with other, nonfunctional foods as a dietary supplement.

One of the best known and widely used functional foods is soy. A large body of accumulated evidence suggests that soy consumption may protect against the development of various cancers, and particularly hormone-dependent cancers, such as breast cancer, prostate cancer, and uterine cancer. For example, breast cancer is the second most common cancer in women, and is the second leading cause of cancer death among women. Asian women who consume a traditional diet high in soy products have a low incidence of breast cancer. However, Asian women who emigrate to the West and subsequently adopt a Western diet characterized by relatively low soy consumption present increased rates of breast cancer. Soy

consumption is also related to decreased rates of other cancers, such as rectal and colon cancer.

Soy contains five classes of compounds that have been identified as having anticarcinogenic properties. These are isoflavones, saponins, phytates, protease inhibitors, and phytosterols. Soy is the only significant source of isoflavones. It has been suggested that the reduced rates of cancer development associated with soy consumption may be due to the presence in soy of various isoflavones, which can be converted in the body to e.g., biochanin A, diadzein, genistin, genistein, and coumestrol, which are weakly estrogenic and anticarcinogenic. For example, the isoflavone genistein has been shown to suppress the growth of a wide range of cancer cells. Genistein is a specific inhibitor of protein tyrosine kinases, and also inhibits DNA topoisomerase activity and other proteins involved in signal transduction pathways. The other anticarcinogens found in soy are believed to exert their beneficial effects in a variety of ways that are not as well studied as those of the isoflavones. Saponins and phytates are thought to be antioxidants, which protect against the development of tumor-promoting free radicals. Protease inhibitors, such as the Bowman-Birk inhibitor, are found in soybeans in only small amounts and apparently prevent the activation of oncogenes, which can lead to transformation of normal cells to cancerous cells. Phytosterols have been shown in animal models to inhibit cancer development, although the mechanism is not at all clear.

In addition to its potent anticarcinogenic activity, soy has also been shown to inhibit development of osteoporosis. Soy protein was shown to reduce the onset of bone loss in aging rats, and this correlated with a decline in the rate of increase of serum parathyroid hormone levels with age. A similar result was seen in rats fed the isoflavone genistein alone. Bone resorption was inhibited in postmenopausal women given ipriflavone, a drug that is



metabolized to daidzein, another isoflavone found in soybeans. Furthermore, soy is a good source of calcium, and the bioavailability of calcium from soybeans is equivalent to that of milk. In addition, humans consuming soy protein lose less calcium in their urine than humans consuming animal protein.

5 Soy contains a number of nutrients that can help fight the development of cardiovascular disorders. Soy is low in saturated fat (about 85% of the fat in soybeans is unsaturated). Soy oil is rich in the essential fatty acids linoleic acid and linolenic acid. One essential fatty acid found in soy,  $\alpha$ -linolenic acid, is associated with a lower risk of stroke. Soy lecithin has been shown to lower cholesterol in normal rats, and soy phytosterols lower  
10 total and LDL (low-density lipoprotein) cholesterol and improve the ratio of HDL (high-density lipoprotein) to LDL cholesterol in healthy human subjects. Soy fiber has also been shown to help lower total and LDL cholesterol levels in patients with high cholesterol levels. Soy protein has also been shown to reduce serum cholesterol levels in hypercholesterolemic subjects. However, soy protein does not appear to have a significant effect on the serum  
15 cholesterol levels of normal subjects, and the mechanism by which soy effects changes in cholesterol levels is not completely understood.

It is well known that functional food preparations can vary in the amount of various nutrients over a wide range. For example, the content of phytoestrogens in soy differs depending on the form of the soy product, where the plants are grown, the weather  
20 conditions, and the maturation stage. One study (Franke et al., *J Ag Food Chem* 1994 42:1905-1913) showed that where all the isoflavones were converted by acid hydrolysis to their aglycones, the levels of genistein varied between 430 mg/kg and 2,040 mg/kg, depending on the source of the soy. Soy oil is higher than other soyfoods in essential fatty acid content and vitamin E. Soyfoods made from whole soybeans, such as tempeh, miso and

natto, are high in fiber, while others, such as soy oil, soy milk and tofu, contain very little fiber. As a result, different soy foods may have very different health benefits.

The physiological effect of a given amount of a functional food bioactive component is dependent on a number of factors in addition to the amount of that substance ingested.

- 5 Such factors include bioavailability and the presence or absence of interactions among components of the functional food. With respect to these interactions, some may enhance the physiological effect of the active components and others may detract from the physiological effect, by, for example, affecting the bioavailability of the active component. The presence or extent of these interactions may depend on the relative or absolute concentrations of the
- 10 various components. These interactions may produce difficulties in identifying the active component(s) that contribute to the physiological effect, because the presence or absence, or the concentrations, of the other functional food components interacting with the active component might affect the bioactivity or bioavailability of the active component.
- Furthermore, even if the active component(s) that contribute to the physiological effect can
- 15 be identified, the influence of the other interacting component(s) may complicate the characterization of the bioactivity of the identified active component(s). Standardization of a functional food product or dietary supplement is therefore sensitive to the presence or absence of these interactions, and therefore depends on the ability of the manufacturer to detect, measure and control for the interactions of these components, active and/or inactive.
- 20 However, functional foods, when they are standardized at all, are usually standardized to contain a certain level of a small number of ingredients or components. As a result, these functional food preparations may vary dramatically in the content of one or more active components, and further still may vary dramatically in the physiological effect associated with those active components, due to a lack of appreciation of the interactions among the

functional food components. Furthermore, variations in the procedures used to prepare the functional food product may result in loss or gain of one or more components, which may influence the bioactivities associated with that functional food product. Therefore, there is a need for a method to ensure a functional food product or dietary supplement that contains a  
5 consistent level of bioactivity associated with desired health benefits.

### SUMMARY OF THE INVENTION

The methods of the present invention provides a standardized functional food product or dietary supplement associated with desired health benefits. One or more extracts,  
10 preferably derived from a botanical source such as soy beans, are assayed in one or more bioassays associated with a desired physiological response or health benefit to detect one or more bioactivities of the total extract. Known or suspected chemical constituents of the extract, known or suspected to be associated or correlated with the desired physiological response or health benefit, may also be assayed to determine if the bioactivity (or  
15 bioactivities) of the extract may be due to these known constituents. The extract(s) may also be fractionated to detect active components that may be responsible for the activity of the extract. The amounts and/or the bioactivities of the active components in the extract are then determined. The determination of the amounts and/or the bioactivities of the active components, as well as the total extract bioactivities, allows the development of a functional  
20 food product standard associated with a desired physiological response or health benefit against which other preparations of the same functional food product can be tested. Thus, a functional food product or dietary supplement having desirable health benefits may be prepared with an assured level of activity.

Therefore, it is an object of the present invention to provide a method for standardizing a botanical extract to create a standardized functional food product or a standardized dietary supplement, comprising the steps of removing at least one first aliquot from the extract; determining from the first aliquot a bioactivity value for the extract, using  
5 an extract bioassay to measure the bioactivity value; comparing the bioactivity value for the extract to a bioactivity standard that specifies one of a minimal total bioactivity, a maximal total bioactivity, and a range of total bioactivities required for a standardized functional food product or a standardized dietary supplement as measured using the extract bioassay to provide a bioactivity comparison; removing at least one second aliquot from the extract;  
10 fractionating the second aliquot into a plurality of fractions, the plurality of fractions containing one or more active components; determining from among the plurality of fractions the amount in the extract of at least one of the one or more active components, the one or more active components having activity in an active component bioassay; comparing the amount of the one or more active components determined above to an active component  
15 standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal amount, a maximal amount, and a range of amounts of the at least one of the one or more active components to provide a quantitative compositional fingerprint comparison; and determining whether the extract is a standardized functional food product or a standardized dietary supplement based on the bioactivity comparison and the  
20 quantitative compositional fingerprint comparison.

Variations of this procedure are also contemplated as being within the scope of the present invention. For example, the present invention also provides a method for standardizing a botanical extract to create a standardized functional food product or a standardized dietary supplement, comprising the steps of removing at least one first aliquot

from the extract; determining from the first aliquot a bioactivity value for the extract, using an extract bioassay to measure the bioactivity value; comparing the bioactivity value for the extract to a bioactivity standard that specifies one of a minimal total bioactivity, a maximal total bioactivity, and a range of total bioactivities required for a standardized functional food product or a standardized dietary supplement as measured using the extract bioassay to  
5 provide a bioactivity comparison; removing at least one second aliquot from the extract; fractionating the second aliquot into a plurality of fractions, the plurality of fractions containing one or more active components; determining from among the plurality of fractions the bioactivity in the extract of at least one of the active components having activity in an active component bioassay; comparing the bioactivity of the one or more active components  
10 determined above to an active component bioactivity standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of the one or more active components to provide a component bioactivity fingerprint comparison; and determining  
15 whether the extract is a standardized functional food product or a standardized dietary supplement based on the bioactivity comparison and the component bioactivity fingerprint comparison.

In addition, the present invention provides a method for standardizing a botanical extract to create a standardized functional food product or a standardized dietary supplement,  
20 comprising the steps of removing at least one first aliquot from the extract; determining from the first aliquot a bioactivity value for the extract, using an extract bioassay to measure the bioactivity value; comparing the bioactivity value for the extract to a bioactivity standard that specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities required for a standardized functional food product or a standardized dietary supplement as

measured using the bioassay to provide a bioactivity comparison; removing at least one second aliquot from the extract; fractionating the second aliquot into a plurality of fractions, the plurality of fractions containing one or more active components; determining from among the plurality of fractions the bioactivity and the amount in the extract of at least one of the active components having activity in the an active component bioassay; comparing the amount of the one or more active components determined above to an active component standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal amount, a maximal amount, and a range of amounts of the active components to provide a quantitative compositional fingerprint comparison;

5 comparing the bioactivity of the active components determined above to an active component bioactivity standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of the one or more active components to provide a component bioactivity fingerprint comparison; and determining whether the extract is a standardized functional food

10 product or a standardized dietary supplement based on the bioactivity comparison, the component bioactivity fingerprint comparison, and the quantitative compositional fingerprint comparison.

15

In another aspect, the present invention provides a method for establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, comprising the steps of establishing a botanical extract; fractionating an aliquot of the extract into a plurality of fractions, the plurality of fractions containing substantially all the mass of the aliquot; ascertaining an amount of at least one active component in at least one of the plurality of fractions; specifying an active component standard for the fingerprint standard, the active component fingerprint standard including one of a minimal amount, a

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maximal amount, and a range of amounts of the active component in the plurality of fractions; ascertaining a bioactivity value of the extract, using an extract bioassay; specifying a bioactivity standard for the fingerprint standard, the bioactivity standard including one of a minimal bioactivity value, a maximal bioactivity value, and a range of bioactivity values in the extract for the extract bioassay; and establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, the fingerprint standard comprising the active component standard and the bioactivity standard.

In another aspect, the present invention provides a method for establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, comprising the steps of establishing a botanical extract; fractionating an aliquot of the extract into a plurality of fractions, the plurality of fractions containing substantially all the mass of the aliquot; ascertaining a bioactivity of at least one active component in at least one of the plurality of fractions; specifying an active component bioactivity standard for the fingerprint standard, the active component bioactivity standard including one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of the active component in the plurality of fractions; ascertaining a bioactivity value of the extract, using an extract bioassay; specifying a bioactivity standard for the fingerprint standard, the bioactivity standard including one of a minimal bioactivity value, a maximal bioactivity value, and a range of bioactivity values in the extract for the extract bioassay; and establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, the fingerprint standard comprising the active component bioactivity standard and the bioactivity standard.

In another aspect, the present invention provides a method for preparing a standardized functional food product or a standardized dietary supplement, comprising the

steps of preparing a plurality of extracts from at least one botanical; screening each of the plurality of extracts against a plurality of bioassays, each of the plurality of bioassays being associated with a target physiological condition; identifying one or more of the plurality of extracts having a desired level of bioactivity in at least one of the plurality of bioassays;

5 determining the level of bioactivity in each of the plurality of extracts identified above for each of the plurality of bioassays; and mixing two or more of the plurality of extracts to form a standardized functional food product or a standardized dietary supplement, the standardized functional food product or standardized dietary supplement having a desired level of bioactivity for each of the plurality of bioassays associated with the target physiological

10 condition.

The bioassays of the total extract may be the same as or different from those used in measuring the bioactivity in the fractions or the active components. Furthermore, the samples may be fractionated until only a single type of molecule is obtained, or the fractionation may be performed only until a mixture or a class of components is obtained.

15 The methods of the present invention are performed at least once during the preparation of the standardized functional food product or dietary supplement. However, the method may be performed more than once and may in fact be performed reiteratively throughout the whole process, as many times as desired. Multiple tests are preferred because the characteristics of the functional food product may change during processing, and such

20 reiterations ensure the quality of the food product. If at any time the functional food product preparation fails the standardization test(s), the preparation may be discarded. Alternatively, the functional food product may be modified and thereafter retested to determine whether the modified functional food product now passes the standardization test(s).



In another embodiment of the present invention, an extract derived from a functional food product source may be surveyed in a large number of bioassays correlated with a variety of different diseases or conditions in order to determine as many of the potential bioactivities of the extract as possible. For example, extracts of ground soybeans may be assayed to detect  
5 bioactivities related to specific physiological conditions, such as cancer, osteoarthritis, cardiovascular disorders, menopause, diabetes, nutritional imbalances, and food allergies. In addition, fractions or known components of the extract may be assayed in the same assays, to determine if these fractions or components are responsible for the bioactivities detected in the extracts. The bioactivities of the extracts, the fractions and the known components may be  
10 quantified, and thereafter the bioactivities of the fractions or known components may be compared to that of the extract to determine if all the bioactivities are accounted for by the fractions or the known components. In this fashion, unexpected interactions, such as synergies or masking, may be detected. Once such a survey of the bioactivities of the extract is completed, the same survey may be performed on commercially prepared functional food  
15 products, derived from the same functional food product source, to detect bioactivities that are no longer present in the functional food product. In this fashion, desired components and/or bioactivities lost during preparation of the functional food product may be identified and the measures undertaken to replace some or all of these bioactivities. Alternately, the procedures employed in preparation of the functional food product may be altered to diminish  
20 or eliminate the loss of desired components and/or bioactivities, if these activities were not desired in the that particular functional food product. In addition, different extracts, functional food products or dietary supplements may be blended to achieve a desired combination of bioactivities that are targeted to a specific physiological condition.

The above and other objects of the present invention will become apparent from the following description.

### BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1A is a bar chart that illustrates the inhibition of various bioassays (expressed as a percent of full activity) by soy full-fat flakes (open bars) and soy defatted flakes (shaded bars). For each assay, the soy full-fat flakes and soy defatted flakes were tested at 1 mg/ml. Bioassay 1, adrenergic  $\alpha 1$  receptor binding, NS; bioassay 2, adrenergic  $\alpha 2$  receptor binding, NS; bioassay 3, adrenergic  $\beta$  receptor binding, NS; bioassay 4, dopamine D2 receptor  
 10 binding; bioassay 5, EGF receptor binding; bioassay 6, EGF receptor tyrosine kinase activity; bioassay 7, ERK serine/threonine kinase activity; bioassay 8, estrogen receptor  $\alpha$  binding; bioassay 9, estrogen receptor  $\beta$  binding; bioassay 10, glucocorticoid receptor binding; bioassay 11, HER2 tyrosine kinase activity; bioassay 12, HMG CoA reductase activity; bioassay 13,  $\text{Ca}^{++}$ -dependent  $\text{K}^+$  channel binding; bioassay 14, IL-6 receptor binding;  
 15 bioassay 15, IL-8 receptor binding; bioassay 16, lipid peroxidation; bioassay 17, MAP kinase activity; bioassay 18, constitutive nitric oxide synthase activity; bioassay 19, oxytocin receptor binding; bioassay 20, p59<sup>lyn</sup> tyrosine kinase; bioassay 21, p56<sup>lck</sup> tyrosine kinase; bioassay 22, PAF receptor binding; bioassay 23, progesterone receptor binding; bioassay 24, protein kinase A activity, NS; bioassay 25, superoxide dismutase activity; bioassay 26, testosterone receptor binding; bioassay 27, thromboxane A<sub>2</sub>-induced platelet aggregation;  
 20 bioassay 28, thyrotropin-releasing hormone receptor binding; bioassay 29, TNF receptor binding; bioassay 30, xanthine oxidase activity.

Figure 1B is a bar chart that illustrates the amounts of various chemical constituents detected in soy full-fat flakes (open bars) and soy defatted flakes (shaded bars). The

concentrations are expressed in ppm. A: daidzein; B; genistein; C: glycitein; D: daidzin; E: genistin; F: glycitin; G: 6-O-acetyl daidzin; H: 6-O-acetyl genistin; I: 6-O-acetyl glycitin; J: 6-O-malonyl daidzin; K: 6-O-malonyl genistin; L: 6-O-malonyl glycitin; M: L- $\alpha$ -lysophosphatidylcholine; N: L- $\alpha$ -phosphatidylcholine; O: L- $\alpha$ -phosphatidylethanolamine; P: L- $\alpha$ -phosphatidylinositol; Q: L- $\alpha$ -phosphatidylserine; R: campesterol; S:  $\beta$ -sitostanol; T:  $\beta$ -sitosterol; U: stigmasterol; V: coumestrol; W:  $\alpha$ -tocopherol; X: Bowman-Birk trypsin inhibitor (BBI). In the cases of A, B, and C, the concentrations shown are 10x those actually detected; for M, N, O, P, Q, and X, the concentrations shown are 1/10<sup>th</sup> those actually detected.

10        Figures 2A-2F show the degree of inhibition by different soy extracts of various bioassays associated with osteoporosis. Figure 2A, inhibition of estrogen receptor  $\beta$  binding by aqueous soy extracts. Figure 2B, inhibition of p59<sup>l<sup>n</sup></sup> tyrosine kinase activity by aqueous soy extracts. Figure 2C, inhibition of estrogen receptor  $\beta$  binding by dimethyl sulfoxide (DMSO) soy extracts. Figure 2D, inhibition of p59<sup>l<sup>n</sup></sup> tyrosine kinase activity by DMSO soy  
15        extracts. Figure 2E, inhibition of estrogen receptor  $\beta$  binding by ethanol soy extracts. Figure 2F, inhibition of p59<sup>l<sup>n</sup></sup> tyrosine kinase activity by ethanol soy extracts.

      Figures 3A-3F show the degree of inhibition by different soy extracts of various bioassays associated with osteoporosis. Figure 3A, inhibition of p56<sup>l<sup>ck</sup></sup> tyrosine kinase activity by aqueous soy extracts. Figure 3B, inhibition of EGF receptor binding activity by aqueous soy extracts. Figure 3C, inhibition of p56<sup>l<sup>ck</sup></sup> tyrosine kinase activity by DMSO soy  
20        extracts. Figure 3D, inhibition of EGF receptor binding activity by DMSO soy extracts. Figure 3E, inhibition of p56<sup>l<sup>ck</sup></sup> tyrosine kinase activity by ethanol soy extracts. Figure 3F, inhibition of EGF receptor binding activity by ethanol soy extracts.

Figures 4A-4B show the degree of inhibition by different soy extracts of various bioassays associated with cardiovascular diseases. Figure 4A, inhibition of estrogen receptor  $\alpha$  binding by aqueous soy extracts. Figure 4B, inhibition of estrogen receptor  $\alpha$  binding by ethanol soy extracts.

5        Figures 5A-5F show the degree of inhibition by different soy extracts of various bioassays associated with cardiovascular diseases. Figure 5A, inhibition of HMG CoA reductase activity by aqueous soy extracts. Figure 5B, inhibition of lipid peroxidation by aqueous soy extracts. Figure 5C, inhibition of HMG CoA reductase activity by DMSO soy extracts. Figure 5D, inhibition of lipid peroxidation by DMSO soy extracts. Figure 5E,  
10       inhibition of HMG CoA reductase activity by ethanol soy extracts. Figure 5F, inhibition of lipid peroxidation by ethanol soy extracts.

Figures 6A-6E show the degree of inhibition by different soy extracts of various bioassays associated with cardiovascular diseases. Figure 6A, inhibition of PAF receptor binding by aqueous soy extracts. Figure 6B, inhibition of nitric oxide synthetase activity by  
15       aqueous soy extracts. Figure 6C, inhibition of PAF receptor binding by DMSO soy extracts. Figure 6D, inhibition of PAF receptor binding by ethanol soy extracts. Figure 6E, inhibition of nitric oxide synthetase activity by ethanol soy extracts.

Figures 7A-7E show the degree of inhibition by different soy extracts of various bioassays associated with cardiovascular diseases. Figure 7A, inhibition of thromboxane A<sub>2</sub>  
20       platelet aggregation by aqueous soy extracts. Figure 7B, inhibition of superoxide dismutase activity by aqueous soy extracts. Figure 7C, inhibition of thromboxane A<sub>2</sub> platelet aggregation by DMSO soy extracts. Figure 7D, inhibition of superoxide dismutase activity by DMSO soy extracts. Figure 7E, inhibition of thromboxane A<sub>2</sub> platelet aggregation by

ethanol soy extracts. Figure 7F, inhibition of superoxide dismutase activity by ethanol soy extracts.

Figures 8A-8C show the degree of inhibition by different soy extracts of various bioassays associated with postmenopausal symptoms. Figure 8A, inhibition of adrenergic  $\alpha 2$  activity by aqueous soy extracts. Figure 8B, inhibition of adrenergic  $\alpha 2$  activity by DMSO soy extracts. Figure 8C, inhibition of adrenergic  $\alpha 2$  activity by ethanol soy extracts.

Figures 9A-9F show the degree of inhibition by different soy extracts of various bioassays associated with breast cancer prevention. Figure 9A, inhibition of HER2 tyrosine kinase activity by aqueous soy extracts. Figure 9B, inhibition of MAP kinase activity by aqueous soy extracts. Figure 9C, inhibition of HER2 tyrosine kinase activity by DMSO soy extracts. Figure 9D, inhibition of MAP kinase activity by DMSO soy extracts. Figure 9E, inhibition of HER2 tyrosine kinase activity by ethanol soy extracts. Figure 9F, inhibition of MAP kinase activity by ethanol soy extracts.

Figures 10A-10C show the degree of inhibition by different soy extracts of various bioassays associated with breast cancer. Figure 10A, inhibition of EGF receptor binding by aqueous soy extracts. Figure 10B, inhibition of EGF receptor binding by DMSO soy extracts. Figure 10C, inhibition of EGF receptor binding by ethanol soy extracts.

Figures 11A-11F show the degree of inhibition by different soy extracts of various bioassays associated with premenstrual tension syndrome. Figure 11A, inhibition of oxytocin receptor binding by aqueous soy extracts. Figure 11B, inhibition of progesterone receptor binding by aqueous soy extracts. Figure 11C, inhibition of oxytocin receptor binding by DMSO soy extracts. Figure 11D, inhibition of progesterone receptor binding by DMSO soy extracts. Figure 11E, inhibition of oxytocin receptor binding by ethanol soy extracts. Figure 11F, inhibition of progesterone receptor binding by ethanol soy extracts.

Figures 12A-12D show the degree of inhibition by different soy extracts of various bioassays associated with premenstrual tension syndrome. Figure 12A, inhibition of dopamine D2 receptor binding by aqueous soy extracts. Figure 12B, inhibition of thyrotropin releasing hormone receptor binding by aqueous soy extracts. Figure 12C, inhibition of dopamine D2 receptor binding by ethanol soy extracts. Figure 12D, inhibition of thyrotropin releasing hormone receptor binding by ethanol soy extracts.

Figure 13 illustrates the preferred ranges of bioactivities about the  $IC_{50}$  values associated with the total bioactivity standards of a preferred embodiment of the present invention.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides a method, the PharmaPrint™ process, by which a standardized unctonal food product or dietary supplement may be made. One aspect of the present invention involves a method to establish a standardized fingerprint of the functional food or dietary supplement. Another aspect of the present invention involves a method to prepare a standardized preparation of the functional food or dietary supplement, based on comparison of a sample of the functional food product to the standardized fingerprint for that functional food product. Another aspect of the present invention relates to a method for surveying an extract of a functional food product source, such as soy, for bioactivities related to a number of different diseases and conditions, and comparing the total activities of the extract to those of a functional food product in order to identify any bioactivities lost during preparation of the functional food product, or to design extracts lacking unwanted biological activities.

As used herein, the term "component" refers to a discrete chemical compound, or class of chemical compounds, which either is present naturally in the functional food or the source of the functional food, or which has been added to the functional food. An "active component" is a component, or a group of closely related components, which exhibits a certain minimal level of activity in an assay of biological activity that is related to an effect, beneficial or otherwise, that the functional food has upon a cell or an organism, i.e., a human or an animal. Activity includes enzyme activity, receptor binding, or any change in a measured parameter that is associated with an effect that the functional food has on a cell or an organism. An "inactive component", conversely, is a component, or a group of closely related components, which fails to exhibit a certain minimal level of activity in an assay of biological activity that is related to an effect, beneficial or otherwise, that the functional food has upon an organism. "Fraction" refers to a unit portion of an extract that is produced by a separation procedure employed in detecting and/or identifying a bioactivity, a component or an active component in an extract of the functional food product. A fraction is usually a product of selective solute solubility and/or partition techniques, including but not limited to liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, and chromatographic separation techniques, such as flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

The term "fingerprint" refers to specified levels or ranges of concentration and/or bioactivity for specified elements comprising the reference standard used to standardize the functional food product or dietary supplement. The elements may be, for example, an

extract, a fraction, an active component, or an inactive component. The fingerprint may specify an extract of a functional food source that exhibits certain defined absolute and/or relative levels of bioactivity. In addition, the fingerprint may specify a fraction or a particular active component or class of components having certain defined absolute and/or relative levels of bioactivity. Furthermore, the fingerprint may specify certain biologically active or inactive components in a standardized functional food product or dietary supplement that are within specified absolute and/or relative concentration or activity ranges. The bioactivity is usually and preferably, but not necessarily, measured in an assay of biological activity that is related to an effect, beneficial or otherwise, that the functional food product has upon an organism, i.e., a human or an animal.

To establish the fingerprint for the functional food product of interest, an extract of the functional food product source is first prepared. The functional food product source, usually a plant product, is first mechanically homogenized, usually by grinding, chopping, or the like. While usually of plant origin, the functional food product source may also be of, for example, animal, fungal, or microbial origin, or from expression in plants, animals or microbial organisms, using recombinant DNA techniques. The resulting product may then be used as the functional food extract, or alternately may be further processed. For example, the homogenized product may be extracted with an aqueous or organic solvent or mixture of solvents. Alternatively, the homogenized product may be heated or cooled, or treated with a digestive chemical or enzyme. However it is prepared, the extract should be produced in such a way that the bioactivity or bioactivities of interest are retained in the extract.

The extract is then assayed to detect the presence of a bioactivity of interest. The bioactivity is typically measured in a bioassay, and preferably in a bioassay that measures either an effect on an enzyme activity or an effect on a receptor binding activity. Where the



bioassay is used to measure a bioactivity present in the extract, it may be referred to herein as an extract bioassay. The use of bioassays that rely on the measurement of effects on whole organisms or organ systems of whole organisms *in vivo* is not preferred, since these assays are typically more expensive, time-consuming and less reliable than enzyme or receptor

5 bioassays. A bioactivity value of the extract is measured using the extract bioassay to determine if, for example, the extract is effective to inhibit (or enhance) the enzyme activity toward a substrate, or if the extract is effective to block (or enhance) the binding and/or uptake of a ligand for the receptor. The level of bioactivity of the extract, where present, is quantified by standard procedures known to those in the art, e.g., comparison to a parallel

10 assay run in the absence of the extract, or by comparison to a standard curve that relates the level of a quantified chemical compound to a level of bioactivity.

Regardless of whether an extract exhibits bioactivity in an extract bioassay, the extract may be further separated into fractions. Fractionation may be performed by any appropriate technique, including but not limited to liquid/liquid extractions, pH-dependent

15 separations, isoelectric focusing, preparative electrophoresis, and chromatographic separation techniques, such as flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography, and any

20 other techniques generally known to those of skill in the art. The separated fractions may also be assayed in a bioassay in which the extract exhibits activity to determine if all or a portion of the bioactivity of the extract is contained in the fractions. The fractions may be further fractionated until one or more fractions contain only a single class of molecules, or even only a single, isolated and essentially purified chemical compound that has activity in

the bioassay of interest. Known chemical constituents of the functional food product may also be bioassayed, in a manner similar to that described above. The activity (or activities) of the known chemical constituents in the bioassay, if present, may assist in identifying the compounds in the functional food product responsible for the activity in the extract. The single class or the purified chemical compound(s) may then be identified and quantified, using standard chemical or physical techniques, to obtain a value for the concentration of the active component in the extract, and/or in the fraction. Alternatively, the bioactivity of the active component may be determined. A bioassay that measures the bioactivity of an active component may be referred to herein as an active component bioassay. Concentrations of inactive components may also be determined, since these may be employed to act as internal standards that indicate the presence of other active components, or they may act to modulate the activity of the active components.

In addition, the extract may be further fractionated, using an additional separation method or methods distinct from the first separation method(s), to detect other components or groups of components having activity in the same bioassay. The new fractions containing the activity of interest may be still further fractionated, as described above, until one or more fractions contain only a single class of molecules, or even only a single, isolated and essentially purified chemical compound that has activity in the bioassay of interest.

Fractions containing bioactivity may lose activity or prove inactive upon further fractionation. Alternatively, fractions may demonstrate increased bioactivity, or inactive fractions may gain bioactivity upon further fractionation. Consequently, such fractionation procedures are of potential importance in identifying active components that are not apparent upon initial fractionation. Furthermore, these procedures may identify fractions or components that are of potential use in standardization procedures. It is also possible to

standardize against fractions, as opposed to specific compounds or classes of compounds, without identifying the individual components involved in the bioactivity.

The process of bioassay of the extract, accompanied by fractionation of the extract into fractions containing components having bioactivity in the assay, may be repeated as many times as desired, using other bioassays of interest. Thereafter, the active and/or inactive components may be identified and quantified to obtain a value for the concentrations of these components in the extract or fractions. In this fashion, a panel of enzyme activities associated with the extract as well as fractions of the extract and, as desired, with isolated active components of the extract, may be constructed. The panel may also contain the concentrations of the active and or the inactive components, either in the extract or in the fraction from which the active component(s) bioactivity is measured.

The functional food fingerprint standard is established by setting an acceptable minimal, maximal, or ranges of bioactivity values of either the extract, or the active components, or fractions containing bioactivity, or any combination of these to create a bioactivity fingerprint standard. These bioactivity values in the bioactivity fingerprint standard are primarily determined as those that provide for the desired levels of bioactivity of the processed functional food product or dietary supplement as a whole in the organism, be it human or animal, which ingests the functional food product. In addition, acceptable minimal, maximal, or ranges of amounts or concentrations of active and/or inactive components in the extract, termed the quantitative compositional fingerprint standard, may be set as another element of the functional food fingerprint standard. These minimal, maximal, or ranges of values, like the bioactivity concentrations or amounts, are primarily determined as those that provide for the desired levels of bioactivity or concentration of the active component of the processed functional food product or dietary supplement as a whole in the organism which

ingests the functional food product. Thus, the functional food fingerprint standard may comprise, for example and without limitation, measurements of the total bioactivity and the concentrations of one or more active or inactive components; measurements of the total bioactivity and the bioactivity of one or more active components; measurement of the concentrations and the bioactivities of one or more active components; or measurements of the total bioactivity, the bioactivity of one or more active components, and the concentrations of one or more active and/or inactive components. The bioactivity of one or more fractions may be measured and included in addition to or as a replacement for measurement of the bioactivity of either the total extract or one or more active components. Other combinations would be evident to the person of ordinary skill in the art and could be employed where appropriate.

For the methods of the present invention, it is desirable that the concentration of one or more active components falls within a concentration range that produces between 0.1 % to 99.9% of the bioactivity measured in the total extract. More preferably, this concentration range is one that produces between 25% to 85% of the bioactivity, and most preferably 35% to 66% of the bioactivity. As shown in Figure 13, these ranges encompass the  $IC_{50}$  concentrations plus varying inhibitory concentrations higher and lower than the  $IC_{50}$ . Alternatively, the concentration range for the fingerprint could be set such that the range is within 10x the lowest concentration at which saturation occurs and 0.1x the lowest concentration at which measurable specific activity (i.e., bioactivity above background levels, or bioactivity above that measured in the absence of the active component) is detected. Such a concentration range could be useful where, for example, the activity of the active component is masked in the total extract or where the extract exhibits synergy. Alternatively,

the range could be open-ended, with a minimal or maximal bioactivity established as the starting point.

To prepare a standardized functional food product, a sample or a fraction of the functional food is assayed for bioactivity in the assay(s) that compose the bioactivity fingerprint standard. In addition, the concentrations of the active and/or inactive components that comprise the quantitative compositional fingerprint standard are also measured. The results of both measurements are then compared to those of the functional food fingerprint standard, forming bioactivity fingerprint comparisons and quantitative compositional fingerprint comparisons, respectively. If the bioactivities and the active and/or inactive component concentrations of the functional food product are within the ranges established in the bioactivity fingerprint standard and the quantitative compositional fingerprint standard, the functional food product sample may be considered to be standardized according to the functional food fingerprint standard. If, however, the bioactivities and the active and/or the inactive component concentrations fall outside these ranges, then the functional food product sample may be considered to not be standardized according to the functional food fingerprint standard.

Those samples that fail the standardization tests may be modified and thereafter retested to determine if the modified sample now may be considered to be standardized according to the functional food fingerprint standard. Such modifications may include, for example, supplementation with one or more active or inactive components, or mixing with one or more other functional food product sample(s) such that the combination of the samples produces a sample that fulfills all the criteria of the functional food fingerprint standard. If the modification produces a sample that that satisfies all the parameters of the functional food

fingerprint standard, the modified sample may be considered to be standardized according to the functional food fingerprint standard.

The tests of the functional food fingerprint standard may be performed at any point in the preparation of the functional food product or dietary supplement, or alternatively may be performed reiteratively throughout the whole preparation process. The latter may be preferable where it is determined that processing results in changes in the composition and/or bioactivity of the product. It may be required that the product pass each test at each step of the preparation process, or that any desired combination of the tests be passed at any step of the process. In any event, at the end of the preparation process, the sample should be able to pass each test in the functional food fingerprint standard to be considered a standardized functional food product.

Where the failure of any of the tests that comprise the functional food fingerprint standard is sufficient to reject the entire sample, then the samples may be pre-screened by performing one or a lesser combination of the tests as a pre-screen. Those samples that pass such a pre-screening step may be further pre-screened, or may thereafter be tested by all the tests of the functional food fingerprint standard. In this fashion, the standardization process may be simplified and the expenses associated with standardization reduced.

It is often the case that various functional food products derived from the same functional food source may contain very different components, and therefore would exhibit widely different bioactivities. For example, different functional foods derived from soybeans have different bioactivities. Soy sauce and soybean oil have virtually no isoflavones, which are compounds that are thought to inhibit the development of some forms of cancer and to decrease the risk of heart disease by helping to lower blood cholesterol levels. In contrast, soyfoods such as tofu, soy milk, soy flour and soy nuts contain isoflavones at concentrations

ranging between 1.3-3.8 mg/g. Soy foods made from whole soybeans, such as tempeh, miso and natto, are high in fiber, while soy milk and tofu contain little or no fiber. Soybean oil has high concentrations of the essential fatty acids, linoleic acid and linolenic acid, but these fatty acids are not found in many other soy foods. In addition, the same functional food product produced by different manufacturers or different manufacturing processes may differ in composition or bioactivities.

As a result, bioactivities present in the functional food product source may be lost during processing to the final functional food product. By surveying the functional food product source, prior to any significant processing, for all the bioactivities associated with that functional food product source, and then assaying the functional food product for the same bioactivities during the production process, the loss of such bioactivities in the final functional food product may be detected. Then, if desired, the components or fractions responsible for some or all of the lost bioactivity or bioactivities may be restored to the functional food product, resulting in an enhanced functional food product. The restoration of the desired bioactivity may be done by adding back to the functional food product an isolated component or family of components responsible for the bioactivity. Alternately, a fraction of the functional food product source containing the components responsible for the bioactivity, or a fraction derived from another functional food product source containing components responsible for a desired bioactivity may be added to the functional food product.

Variations in bioactivity of a functional food product extract may occur as a result of interactions among the various active or inactive components of the soy functional food product. For example, in a given bioassay, the bioactivity of the extract may be greater than the summation of the bioactivities of the individual active components. In this case, the whole is greater than the sum of the parts. This phenomenon is termed "synergy".

Alternatively, in a given bioassay, the bioactivity of the extract may be less than the summation of the bioactivities of the individual active components. Here, the bioactivities of the individual components are inhibited in some way in the extract. This phenomenon is termed "masking". Masking or synergy may occur in multiple bioassays with the same  
5 extract, or masking may occur in some bioassays while synergy is exhibited in other bioassays of the same extract. Bioassays that demonstrate synergy are particularly useful in assays of total extract bioactivity, since these assays are sensitive to the presence (and optionally, the concentration) of a particular set of active and/or inactive components. Bioassays that demonstrate masking are conversely particularly useful in assays of the  
10 bioactivity (or bioactivities) of various active component(s), since these assays assist in detection and/or quantification of various active components that might otherwise go undetected in bioassays of total extracts or certain fractions. Therefore, bioassays that demonstrate synergy and/or masking may be particularly useful in standardizing a functional food product, in that these assays may enable standardization of a functional food product  
15 using fewer tests than would otherwise be required.

Detection of synergy or masking requires determining the amount of the bioactivity in the total extract and comparing that amount to the summation of the bioactivity in all the bioactive fractions, or, alternatively, in all the isolated active components that contribute to the total bioactivity in the assay. To confirm the presence of synergy or masking, the isolated  
20 fractions (or active components) may be recombined and the activity of the recombined sample compared with that of the unfractionated sample. If the activities are the same or substantially similar, then synergy or masking may be occurring. However, if the activity of the recombined sample is substantially less than that of the unfractionated sample, then the diminished activity of the recombined sample may be due to decomposition of the active



components. Various analytical techniques, such as chromatography, may be used to determine if decomposition of the active components is occurring.

Synergistic or masking effects may be due to interactions of active components with one another, or with inactive components in the extracts. Unknown interactions between active and inactive components of the extract may mean that the bioactivity of the extract far exceeds or is far less than that of the individual components, when considered collectively. As a result, to ensure a consistent and complete functional food product, the extract should be assayed to determine the bioactivity in the extract. The bioassays used should be those that measure a bioactivity that is correlated with a specific disease or condition which the functional food product is thought to affect. For example, certain protein tyrosine kinase activities are associated with cancer, a disease for which soy is thought to have a beneficial effect. Inhibition of these enzyme activities may present a way to affect the course of the cancer. Therefore, detection of and standardization against these activities may produce a soy functional food product with consistent beneficial effects.

The present invention is described in terms of the following example, which is intended to be illustrative and is not intended to limit the invention in any way.

Example:

Fresh soybeans were processed by cleaning and flaking in preparation for the extraction process. The flaked soybeans (i.e., soy full-fat flake) were extracted with *n*-hexane resulting in two forms: 1) a solid form (i.e., soy defatted flake) and 2) a soy oil fraction. Soybean extracts used in this study were obtained from Sigma Chemical Corporation (St. Louis, MO, USA).

Whole soybeans were cleaned by soaking in an acetone water mixture (80% acetone, 20% water) with occasional shaking. A sample of cleaned soybeans (400 gms) was weighed out and ground (1 minute, MIX setting) in a small glass blender. The ground soybeans were then passed through a 20-mesh screen. The ground material that passed through the 20-mesh  
5 screen was designated the full-fat soybean flakes.

Full-fat soybean flakes (50.0 gms) were mixed with 200 ml *n*-hexane at room temperature with occasional mixing. After settling for 5 minutes, 120 ml of a hazy, yellow supernatant was decanted and saved. Additional *n*-hexane (120 ml) was added to the solids, and the solids were extracted for a further 30 minutes at room temperature with occasional  
10 mixing. After settling for 5 minutes, a further 100 ml of a light yellow, hazy supernatant was decanted and saved. The heavy solids were slurried and filtered through #54 filter paper under a slight vacuum. The filtered solids were then washed twice with 100 ml of *n*-hexane and sucked dry. The resulting solids (46 gms, approximately 80 ml) were air-dried for one hour to remove the solvent, and then desiccated, 2 hours at 28 inches mercury (Hg) and then  
15 overnight at full vacuum, to produce the soy defatted flakes.

The first and second decants saved from the above extractions was filtered through #54 filter paper. The *n*-hexane washes were added to the filtered decants, and the resulting solution was evaporated under vacuum at 24 inches of Hg. The evaporation was continued in a water bath at 40 °C, and after all the *n*-hexanes evaporated off the evaporation was  
20 continued for an additional 15 minutes at full vacuum. The resulting soy extract oil (9.4 gm) was transferred to an amber bottle and stored under N<sub>2</sub> at 5 °C.

The three soybean extract forms (full-fat flakes, defatted flakes, and extract oil) were subjected to analytical chemical analyses. Elemental composition, H<sub>2</sub>O content, fat content, phospholipid content (measured as total phosphorus, P), isoflavone content, and phytosterol

content determinations were made. Elemental composition and total phosphorus determinations were made by ion-coupled plasma atomic spectroscopy, and fat content was measured by a hot hexane extraction method. Water content was measured by a Karl-Fischer titration method. Isoflavone content was determined by high performance liquid chromatography, and phytosterol content by gas chromatography. The results are shown in Tables 1A-1C. Table 1A shows the results of compositional analyses of different fractions from two different soy preparations, designated A and B. Different preparations of the same material are denoted by a numeral after the letter, i.e., A1, A2, etc.

Table 1A

	Full-fat soybean flakes A1	Defatted soybean flakes A1	Soybean oil A1	Defatted soybean flakes B1	Defatted soybean flakes B2	Defatted soybean flakes B3
Starting weight (wt %)	100	74.6	18.8	---	---	---
H <sub>2</sub> O content (wt %)	10.7	6.3	---	7.7	8.2	8.7
Fat content (wt %)	20	1.7	---	---	---	---
Elemental compo- sition (wt %)						
Ca	0.23	0.32	0.013	0.28	0.37	0.35
K	2.3	3.2	0.04	2.24	2.32	2.30
Mg	0.22	0.30	0.004	0.30	0.34	0.31
P	0.64	0.86	0.054	0.72	0.77	0.74
S	0.34	0.45	0.002	0.38	0.43	0.41
Isoflavone content (ppm)						
Daidzin	240	341	---	996	359	188
Genistin	244	357	---	1,155	418	254
6-O-Malonyl Daidzin	840	1,080	---	1,368	1,427	794
6-O-Malonyl Glycitin	116	153	---	204	176	137
6-O-Malonyl Genistin	1,040	1,370	---	1,662	1,816	1,168
Daidzein	12	16	---	8	11	9
Glycitein	4	6	---	61	35	21
Genistein	13	19	8	13	16	14
Phytosterol content (mg/g)						
$\beta$ -Sitosterol	0.90	0.56	2.28	0.28	0.30	0.31
Campesterol	0.27	0.14	0.77	0.10	0.096	0.096
Stigmasterol	0.24	0.11	0.73	0.066	0.069	0.074

--- : not measured

Referring now to Table 1A, it is evident that the isoflavone constituents partitioned

5 virtually exclusively with the two solid forms (i.e., either full-fat or defatted flake). In contrast, the phytosterols were widely distributed among the different fractions. The total phosphorus measurements indicated that the various forms of phospholipids found in soybeans are located principally in the two solid soy forms.

The results with the defatted flake samples show that the soy samples may vary

10 dramatically in the content of the active components, even when those samples are prepared

in the same manner. The greatest variability is seen in the content of the various isoflavones (Table 1A). For example, the content of 6-O-malonyl daidzin differs between the defatted flake samples by a factor of almost two; the glycitein content among the samples differs by a factor of almost three; there is a four-fold difference in the genistin content, and a five-fold difference in the content of daidzin. As noted previously, others have shown a five-fold difference in the genistein content of soy, depending on various environmental factors.

Tables 1B shows the results of similar compositional analyses of soy solid and oil forms from additional sources. The soy materials were obtained from Cargill Protein Products (Cedar Rapids, IA, USA) or Croda, Inc. (Fullerton, CA, USA). The soy products obtained from Cargill were defatted soy flour samples. Table 1C shows the results of compositional analyses of soy flakes from a variety of sources. The results of Table 1B reinforce and extend those of Table 1A, showing that the isoflavones partition into the solid forms. The results of Table 1C illustrate the variability associated with different sources of the starting material. For example, Novasoy 400 is very high in isoflavone content, while Soyarich B, Procon 2000, and Centroflo 8215 are relatively low. However, the phospholipid content of Novasoy 400 is much lower than that of either Centroflo 8215 or Soyarich B, while the phospholipid content of Procon 2000 is also low. This illustrates the need for standardization of the functional food preparations.

Table 1B

Soy test chemical	Soy Full Flake -P1	Soy Defatted Flake-P1	Soy Full Flake-P2	Soy Defatted Flake- P2	Soy Oil - P2	Soy Oil (Croda)	Soy Flour (Sigma)	Cargill 100/90 Soy Defatted Flake	Cargill 200/70 Soy Defatted Flake
Daidzein	10	8	20	30	<5	<5	50	4700	94
Genistein	10	8	10	20	<5	<5	12	23	35
Glycitein	<5	<5	<5	<5	<5	<5	<10	<5	<5
Daidzin	120	110	260	390	<5	<5	370	530	590
Genistin	98	87	270	410	<5	<5	390	680	800
Glycitin	<5	<5	30	42	<5	<5	140	---	---
6-O-acetyl Daidzin	---	---	---	---	---	---	---	---	---
6-O-acetyl-genistin	---	---	---	---	---	---	---	---	---
6-O-acetyl glycitin	---	---	---	---	---	---	---	---	---
6-O-malonyl Daidzin	---	---	---	---	---	---	---	---	---
6-O-malonyl genistin	---	---	---	---	---	---	---	---	---
6-O-malonyl glycitin	---	---	---	---	---	---	---	---	---
Total isoflavones	200	200	600	900	<5	<5	962	1300	1500
L- $\alpha$ -lyso PC	<500	<500	<1000	<1000	<1000	<12500	<400	<300	<300
L- $\alpha$ -PC	<500	<500	2600	3700	<1000	<12500	4600	2700	2700
L- $\alpha$ -PE	<500	<500	2300	2500	<1000	<12500	600	<300	<300
L- $\alpha$ -PI	1000	<500	<1000	<1000	<1000	<12500	1200	<300	<300
L- $\alpha$ -PS	<500	<500	<1000	<1000	<1000	<12500	<400	<300	<300
Phosphatidic acid	4000	1000	3900	3000	<1000	<12500	900	500	600
Total Phospholipids	5000	1000	11100	11700	<1000	<12500	7300	3200	3300
Campesterol	157	22.5	165	34	720	100	<10	14.7	13.2
$\beta$ -Sitostanol	16.9	<10	20.5	<10	67	20	<10	<10	<10
$\beta$ -Sitosterol	435	101	470	160	1800	550	48	70	52.4
Stigmasterol	134	21.7	145	32	600	77	<10	13.2	12.1
Total Phytosterols	743	149	800.5	232	3190	747	65.5	99.7	79.6
Coumestrol	<100	<1000	0.9	1.3	<10	<10	---	<100	<100
Raffinose	---	---	---	---	---	---	---	15600	16900
Stachyose	---	---	---	---	---	---	---	61900	60200
Tocopherol	15.6	4.1	30.2	5.9	129	28.9	1.2	<2.3	<2.3
Tyrosin Inhibitor	---	---	3508 $\pm$ 2317	9070 $\pm$ 2572	---	---	---	---	---
Total Calcium	---	---	---	---	---	---	---	---	---
Total Potassium	---	---	---	---	---	---	---	---	---
Total Magnesium	---	---	---	---	---	---	---	---	---
Total Phosphorus	---	---	---	---	---	---	---	---	---
Total Sulfur	---	---	---	---	---	---	---	---	---

--- not measured

values are expressed in ppm

P1 - first preparation

P2 - second preparation

Table 1C

Soy test chemical	Supro XT 10c	Supro FXP H1061	Soyarich B	Soyarich I	Novasoy 400	Procon 2000	Soylife 150	Soylife 25	Centraflo 8215
Daidzein	110	220	<5	170	11000	<5	5600	<5	<5
Genistein	34	110	<5	61	2200	<5	260	81	<5
Glycitein	<5	<5	<5	<5	1300	<5	1600	410	<5
Daidzin	320	410	10	380	131000	40	35000	6300	5
Genistin	620	680	20	830	79000	41	9600	1800	6
Glycitin	---	---	---	<5	---	---	---	---	---
6-O-acetyl Daidzin	---	---	---	---	---	---	---	---	---
6-O-acetyl-genistin	---	---	---	---	---	---	---	---	---
6-O-acetyl glycitin	---	---	---	---	---	---	---	---	---
6-O-malonyl Daidzin	---	---	---	---	---	---	---	---	---
6-O-malonyl genistin	---	---	---	---	---	---	---	---	---
6-O-malonyl glycitin	---	---	---	---	---	---	---	---	---
Total isoflavones	1100	1400	40	1400	226000	80	52000	8600	10
L- $\alpha$ -lyso PC	<300	<300	<300	<300	<300	<300	2700	16300	<300
L- $\alpha$ -PC	4100	2800	2000	900	900	2700	41800	32200	6300
L- $\alpha$ -PE	<300	<300	<300	<300	<300	<300	<300	<300	<300
L- $\alpha$ -PI	<300	<300	<300	<300	<300	<300	<300	<300	<300
L- $\alpha$ -PS	<300	<300	<300	<300	<300	<300	<300	<300	<300
Phosphatidic acid	1400	2000	2000	900	700	600	2600	12300	1600
Total Phospholipids	5500	4800	4000	1800	1600	3300	56900	76300	7900
Campesterol	28	158	17	16.3	<10	<10	560	343	293
$\beta$ -Sitostanol	<10	23.3	<10	<10	<10	<10	46	39.8	39.6
$\beta$ -Sitosterol	8608	729	74	79.4	<10	50	3600	2575	658
Stigmasterol	19.8	159	14	13.6	<10	<10	45	245	206
Total Phytosterols	137	1070	107	112	9.2	62	4250	3205	1200
Coumestrol	<100	<100	<0.5	0.77	22	<100	0.82	2.2	<100
Raffinose	2700	1300	--	7200	20300	2600	96100	29800	--
Stachyose	3800	3200	--	6000	31200	18700	108500	72100	15200
Tocopherol	<2.3	<2.3	3.8	<2.3	<2.3	<2.3	214	21.8	21.3
Tyrosin Inhibitor	160.3 $\pm$ 73.6	49.4 $\pm$ 15.4	13.1 $\pm$ 4.3	80.6 $\pm$ 42.4	40.5 $\pm$ 5.9	6.6 $\pm$ 1.9	6.2 $\pm$ 5.7	2.3 $\pm$ 0.23	---
Total Calcium	---	---	---	---	---	---	---	---	---
Total Potassium	---	---	---	---	---	---	---	---	---
Total Magnesium	---	---	---	---	---	---	---	---	---
Total Phosphorus	---	---	---	---	---	---	---	---	---
Total Sulfur	---	---	---	---	---	---	---	---	---

5 Biological Assays

The three forms of soybean extracts (soy full-fat flakes, soy defatted flakes, and soy extract oil) were assayed in a screening panel of biological assays. Each of the solid forms of soy (full-fat flake and defatted flakes) were first extracted with ethanol (E), dimethyl sulfoxide (D), or water (A), then tested in each of the bioassays. For the ethanol extraction procedure, the sample was weighed out, then dissolved in 80% ethanol. The sample was incubated in 80% ethanol for 15 minutes at 45 °C, then the remaining solid portion was spun out into a pellet and the liquid phase taken for testing. For the dimethyl sulfoxide (DMSO) extraction procedure, the sample was weighed out, then dissolved in 100% DMSO. The sample in DMSO was gently mixed (so as to avoid foaming) at room temperature for 5 minutes. The remaining solid portion was spun out as a pellet, and the liquid phase taken for testing. For the aqueous extraction procedure, the sample was weighed out and dissolved in 50 mM Tris buffer, pH 7.2. The sample in Tris buffer was gently vortexed (so as to avoid foaming) for 5 minutes at room temperature. The remaining solid phase was spun out as a pellet, and the liquid phase taken for testing. The various extracts were tested at a concentration of 1.0 mg/ml. In addition, purified forms of known soybean chemical constituents were purchased and tested in the same bioassays to determine which chemical constituents of soybean might be responsible for the activity of the extracts in the bioassays. The chemical constituents were tested at 0.01 mM, 0.03 mM, 0.1 mM or 0.3 mM, based on their formula weights and solubility in the assay. All bioassays were performed at Panlabs, Inc. (Bothell, WA, USA) or CEREP, Inc. (Paris, FRANCE). The bioassay data are shown in Tables 2-5. The darker shaded cells represent  $\geq 50\%$  inhibition of the enzyme or receptor binding activity, and the lighter shaded cells represent between 20% and 50% inhibition.



### Description of Bioassay Procedures

The adrenergic  $\alpha_1$  non-selective assay ("Adrenergic  $\alpha_1$ , NS" in Tables 2-5) [Panlabs assay number 20350] measures binding of [ $^3$ H]-prazosin to adrenergic  $\alpha_1$  receptors. Whole brain (except cerebellum) membranes of male Wistar derived rats weighing  $175 \pm 25$  g are prepared in modified Tris-HCl pH 7.4 buffer using standard techniques. A 5 mg aliquot of membrane is incubated with 0.25 nM [ $^3$ H]-prazosin for 30 minutes at 25 °C. Non-specific binding is estimated in the presence of 0.1  $\mu$ M prazosin. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^3$ H]-prazosin (Greengrass, P. and Bremner, R. Binding characteristics of [ $^3$ H]-prazosin to rat brain  $\alpha$ -adrenergic. Eur. J. Pharmacol. 55: 323-326, 1979). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below. In these and all subsequent tables, the value "nH" refers to the apparent Hill constant.

Assay Parameter	Result
$K_d$	0.09 nM
$B_{max}$	120 fmol/mg protein
Specific Binding	90%

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
Ketanserin	37	9.8	1.2
Phentolamine	44	12	0.8
Prazosin	0.64	0.17	1.0

The adrenergic  $\alpha_2$  non-selective assay ("Adrenergic  $\alpha_2$ , NS" in Tables 2-5) [Panlabs assay number 20390] measures binding of [ $^3$ H]-rauwolscine to adrenergic  $\alpha_2$  receptors. Cerebral cortical membranes of male Wistar derived rats weighing  $175 \pm 25$  g are prepared in modified Hepes pH 7.4 buffer using standard techniques. A 7.5 mg aliquot of membrane is

incubated with 0.7 nM [ $^3$ H]-rauwolscine for 30 minutes at 25 °C. Non-specific binding is estimated in the presence of 1  $\mu$ M yohimbine. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^3$ H]-rauwolscine (Boyajian, C. and Leslie, F.M. Pharmacological evidence for  $\alpha_2$ -adrenoceptor heterogeneity; differential binding properties of tritiated rauwolscine and tritiated idazoxan in rat brain. J. Pharmacol. Exp. Ther. 241: 1092-1098, 1987). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	7 nM
$B_{max}$	250 fmol/mg protein
Specific Binding	80%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
Clonidine	34	31	0.6
Epinephrine	54	49	0.5
Phentolamine	44	40	0.8
Prazosin	270	250	0.8
Yohimbine	19	17	1.0

The adrenergic  $\beta$  non-selective assay ("Adrenergic  $\beta$ , NS" in Tables 2-5) [Panlabs assay number 20430] measures binding of [ $^3$ H]-dihydroalprenolol (DHA) to adrenergic  $\beta$  receptors. Whole brain membranes of male Wistar derived rats weighing  $175 \pm 25$  g are prepared in modified Tris-HCl pH 7.4 buffer using standard techniques. A 7.5 mg aliquot of membrane is incubated with 0.25 nM [ $^3$ H]-DHA for 20 minutes at 25 °C. Non-specific binding is estimated in the presence of 1  $\mu$ M S(-) propranolol. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^3$ H]-DHA (U'Pritchard, D.C., Bylund, D.B. and Snyder, S.H. ((+/-)-[ $^3$ H]-epinephrine and (-)-[ $^3$ H]-

dihydroalprenolol binding to  $\beta_1$  and  $\beta_2$ -noradrenergic receptors in brain, heart, and lung membranes J. Biol. Chem. 253: 5090-5112, 1978). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	0.5 nM
$B_{max}$	83 fmol/mg protein
Specific Binding	85%

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
(-) Alprenolol	0.74	0.49	0.9
(-) Isoproterenol	35	23	0.9
(-) Epinephrine	510	340	0.7

The epidermal growth factor (EGF) binding assay ("EGF" in Tables 2-5) [Panlabs assay number 22550] measures binding of [ $^{125}$ I]-EGF to human EGF receptors. A431 (human epidermoid carcinoma) cells are used to prepare membranes in modified Tris-HCl pH 7.7 buffer using standard techniques. A 5  $\mu$ g aliquot of membrane is incubated with 0.05 nM [ $^{125}$ I]-EGF for 60 minutes at 25 °C. Non-specific binding is estimated in the presence of 10 nM murine EGF. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^{125}$ I]-EGF (Massague, J. Epidermal growth factor-like transforming growth factor: II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. 258: 13614-13620, 1983). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	0.032 nM

$B_{max1}$	1.0 pmol/mg protein
Specific Binding	95%
$K_{d2}$	0.3 nM
$B_{max2}$	4.1 pmol/mg protein

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
EGF (human recombinant)	0.39	0.15	1.2
EGF (murine)	0.4	0.15	0.9
Transforming Growth Factor- $\alpha$ (human)	3.2	1.3	1.2

Binding of EGF or TGF $\alpha$  (transforming growth factor  $\alpha$ ) to the EGF receptor results in activation of intrinsic tyrosine kinase activity. Several cytosolic proteins are phosphorylated by the EGF receptor, leading to activation of signaling pathways that induce mitogenesis and, in some instances, associated cell transformation. Inhibitors of EGF receptor tyrosine kinase activity may be useful for chemotherapeutic intervention in cases of malignant cellular proliferation. The human epidermal growth factor receptor tyrosine kinase assay ("EGF-TK" in Tables 2-5) [Panlabs assay number 17000] involves the use of a recombinantly expressed cDNA encoding the intracellular tyrosine kinase domain of the human EGF receptor (EGF-IKD) cloned and expressed at high levels using a baculovirus expression system in Sf9 insect cells. Test compound and/or vehicle is incubated with the enzyme, immobilized synthetic polypeptide (polyGlu:Tyr 4:1) as substrate and ATP for 10 minutes. Phosphorylated tyrosine residues resulting from activity at the 69 kD kinase domain are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin-linked  $\beta$ -galactosidase enzyme. Fluorescence resulting from conversion of fluorescein-di- $\beta$ -galactoside to fluorescein is measured. The reaction with fluorescein-di- $\beta$ -galactoside is stopped by addition of phenylethyl- $\beta$ -D-thiogalactoside, a

reversible competitive inhibitor of  $\beta$ -galactosidase (Geissler, J.F. *et al.* Thiazolidine-diones: biochemical and biological activity of a novel class of tyrosine protein kinase inhibitors. *J. Biol. Chem.* 265: 22255-22261, 1990). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> ( $\mu$ M)
Staurosporine	6.0
Tyrphostin 47	0.8
Tyrphostin 51	0.31

The extracellular signal-regulated protein kinase (ERK1) is one of the isoforms of mitogen-activated protein kinase (MAP kinase; see below). It is a 42 kD serine/threonine kinase that is activated by dual phosphorylation of tyrosine and threonine residues by the upstream activator MAP kinase. This activation results in the translocation of ERK1 to the cell nucleus where it can phosphorylate a large number of regulatory proteins involved in transcription, translation and cytoskeletal rearrangement. The ERK1 is obtained by the recombinant expression of the cDNA for the human ERK1 serine/threonine kinase in bacterial cells ("ERK1 S/TK" in Tables 2-5) [Panlabs assay number 17100]. Test compound and/or vehicle is incubated with 0.5  $\mu$ g of enzyme, 3.3  $\mu$ g myelin basic protein, [ $\gamma$ -<sup>32</sup>P]-ATP in MOPS buffer, pH 7.2 for 30 minutes at 37 °C. The reaction is terminated by cooling on ice and formation of the <sup>32</sup>P-labeled substrate is measured by scintillation counting (Dudley, D.T. *et al.* A synthetic inhibitor of the mitogen-activate protein cascade. *Proc. Natl. Acad. Sci. USA* 92: 7686-7689, 1995). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> (μM)
2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 098,059)	>1,000
Staurosporine	43

The estrogen receptor  $\beta$  binding assay ("Erb" in Tables 2-5) [Panlabs assay number 22650] measures binding of [<sup>3</sup>H]-estradiol to human ER  $\beta$  receptors. The receptors are obtained from the Panvera Corporation, which owns a stably transfected cell line expressing the human ER  $\beta$  receptor. A 7.5 ng amount of the receptors is incubated with 0.5 nM of [<sup>3</sup>H]-estradiol for 2 hours at 25 °C in a modified Tris-HCL buffer (pH 7.5). Non-specific binding is estimated in the presence of 1 μM diethylstilbestrol. The receptor protein is filtered through a membrane that is washed 3 times and then counted to determine specifically bound [<sup>3</sup>H]-estradiol (Obourn, J.D., Koszewski, N.J. and Notides, A.C. Hormone and DNA binding mechanism of the human estrogen receptor. *Biochemistry*. 32: 6229-6236, 1993). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
K <sub>d</sub>	0.13 nM
B <sub>max</sub>	3,000,000 fmol/mg protein
Specific Binding	90%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
Diethylstilbestrol	0.61	0.13	1.2
17 $\alpha$ -Ethinylestradiol	0.12	0.025	0.9
$\beta$ -Estradiol	0.072	0.015	0.9

The glucocorticoid binding assay ("Glucocorticoid" in Tables 2-5) [Panlabs assay number 23200] measures binding of [<sup>3</sup>H]-dexamethasone to human glucocorticoid receptors. Jurkat (human lymphoma) cells are suspended in modified HEPES pH 7.2 buffer using

standard techniques. Cells ( $6 \times 10^6$ ) are incubated with 20 nM [ $^3\text{H}$ ]-dexamethasone for 120 minutes at 25 °C. Non-specific binding is estimated in the presence of 20  $\mu\text{M}$  dexamethasone. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^3\text{H}$ ]-dexamethasone (Schlechte, J.A. *et al.* Regulation of the glucocorticoid receptor in human lymphocytes. *J. Steroid Biochem.* 16: 69-74, 1982). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	4.3 nM
$B_{\text{max}}$	2,500 receptors/cell
Specific Binding	60%

Compound	$\text{IC}_{50}$ (nM)	$K_i$ (nM)	nH
Cortisol	320	57	0.6
Dexamethasone	38	6.7	0.6
Estrogen	>10,000	-	-
Progesterone	890	160	0.6

10

Human EGF receptor 2 (HER2) tyrosine kinase is an EGF receptor-related protein tyrosine kinase which is overexpressed in many mammary and ovarian carcinomas. Binding of the ligand to the receptor is believed to activate the receptor's intrinsic protein kinase activity, initiating a signal transduction pathway which leads to increase cell proliferation.

15 Truncated HER2, lacking the extracellular and transmembrane regions, is constitutively active. The active kinase transfers  $\gamma$ -phosphates of ATP to the phenolic hydroxyl group of specific tyrosine residues on target proteins. The human HER2 tyrosine kinase assay ("HER2 TK" in Tables 2-5) [Panlabs assay number 17400] involves the use of a recombinantly expressed cDNA produced in Sf9 insect cells using a baculovirus expression system and

purified by affinity chromatography. Test compound and/or vehicle is pre-equilibrated with enzyme and immobilized synthetic polypeptide (polyGlu:Tyr 4:1) as substrate for 15 minutes. Following a 10-minute kinase reaction in the presence of 100  $\mu$ M ATP, phosphorylated tyrosine residues resulting from activity at the 69 kD kinase domain are detected by

5 incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin-linked  $\beta$ -galactosidase enzyme. Fluorescence resulting from conversion of fluorescein-di- $\beta$ -galactoside to fluorescein is measured. The reaction with fluorescein-di- $\beta$ -galactoside is stopped by addition of phenylethyl- $\beta$ -D-thiogalactoside, a reversible

10 competitive inhibitor of  $\beta$ -galactosidase (Bargmann, C.I., Hung, M.C. and Weinberg, R.A. The *neu* oncogene encodes an epidermal growth factor receptor-related protein. Nature 316: 226-230, 1986). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> ( $\mu$ M)
Staurosporine	22
Tyrphostin 47	1.4
Tyrphostin 51	2.5

15 HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A) converts HMG-CoA to mevalonic acid, and is the rate-limiting step in cholesterol biosynthesis. HMG-CoA reductase is isolated from rat liver for this assay ("HMG-CoA" in Tables 2-5) [Panlabs assay number 12400] with the test compound and/or vehicle incubated with [<sup>14</sup>C]-HMG-CoA at 37 °C for 15 minutes. The reaction is then terminated by addition of HCl and [<sup>14</sup>C]-mevalonic

20 acid is separated from the substrate by column filtration and measured by scintillation counting (Kubo, M. and Strott, C.A. Differential activity of 3-hydroxy-3-methylglutaryl



coenzyme A reductase in zones of the adrenal cortex. *Endocrinology* 120: 214-221, 1987).

The reference characteristic for the standard is listed in the table below.

Compound	IC <sub>50</sub> (μM)
Lovastatin	0.012

5        The interleukin-6 (IL-6) assay ("IL-6" in Tables 2-5) [Panlabs assay number 24410] measures binding of [<sup>125</sup>I]-IL-6 to human IL-6 receptors. U266 (human myeloma) cells are the source for the receptor and are tested in a modified HEPES buffer at pH 7.1 using standard techniques. The cells (6.25 x 10<sup>5</sup>) are incubated with 80 pM [<sup>125</sup>I]-IL-6 for 24 hours at 22 °C. Non-specific binding is estimated in the presence of 40 nM IL-6. Cells are

10       centrifuged and pellets are counted to determine specifically bound [<sup>125</sup>I]-IL-6 (Lida, J. *et al.* High affinity interleukin-6 binding sites in bovine hypothalamus. *Eur. J. Pharmacol.* 202: 113-115, 1991). The reference characteristics for the components of the assay and its

15       reference standards are listed in the two tables below.

Assay Parameter	Result
K <sub>d</sub>	0.060 nM
B <sub>max</sub>	670 receptors/cell
Specific Binding	80%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
IL-6	3.2	1.4	0.8

15       The interleukin-8 (IL-8) assay ("IL-8" in Tables 2-5) [Panlabs assay number 24430] measures binding of [<sup>125</sup>I]-IL-8 to human IL-8 receptors. Human neutrophil cells are used to prepare membranes in a modified Tris-HCl pH 7.5 buffer using standard techniques. A 30 μg

20       aliquot of membrane is incubated with 15 pM [<sup>125</sup>I]-IL-8 for 120 minutes at 0 °C. Non-

specific binding is estimated in the presence of 250 nM IL-8. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^{125}$ I]-IL-8 (Grob, P.M. *et al.* Characterization of a receptor for human monocyte-derived neutrophil chemotactic factor/interleukin-8. *J. Biol. Chem.* 265: 8311-8316, 1990). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	1.2 nM
$B_{max}$	200 fmol/mg protein
Specific Binding	85%

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
IL-8	0.68	0.67	0.8

The potassium ( $K^+$ ) channel,  $Ca^{++}$  dependent assay (" $K^+$  channel ( $Ca^{++}$  dependent)" in Tables 2-5) [Panlabs assay number 26580] measures binding of [ $^{125}$ I]-apamin to small conductance calcium-activated potassium channel sites. Whole brain (except cerebellum) membranes of male Wistar derived rats weighing  $175 \pm 25$  g are prepared in a modified Tris-HCl pH 7.4 buffer using standard techniques. A 2.5 mg aliquot of membrane is incubated with 5 pM [ $^{125}$ I]-apamin for 60 minutes at 4 °C. Non-specific binding is estimated in the presence of 100 nM apamin. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^{125}$ I]-apamin (Mourre, C. *et al.* Quantitative autoradiographic mapping in rat brain of the receptor of apamin, a polypeptide toxin specific for one class of  $Ca^{2+}$ -dependent  $K^+$  channels. *Brain Research* 382: 239-249, 1986). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	0.14 nM
$B_{max}$	67 fmol/mg protein
Specific Binding	90%

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
Apamin	0.11	0.11	1.5
Cromokalm	>10,000	-	-
Pinacidil	>10,000	-	-
Nicroandil	>10,000	-	-

Lipid peroxidase stimulation results from a variety of stimuli, including reactive free  
 radicals. Polyunsaturated fatty acids associated with plasma membranes can be degraded due  
 to enzymatic induction of reactive agents such as  $CCl_4$ , leading to cell damage. Microsomes  
 are prepared from rat livers and the protein concentration is determined as the first step of this  
 enzymatic assay ("Lipid peroxidation" in Tables 2-5) [Panlabs assay number 13400]. Test  
 compound and/or vehicle is incubated with 2 mg of the microsomal preparation,  
 polyunsaturated fatty acids, an NADPH generating system and 20 mM  $CCl_4$  for 12 minutes at  
 37 °C. The reaction is terminated by adding a mixture of thiobarbituric acid and  
 trichloroacetic acid. Absorbance proportional to concentration of malondialdehyde is read at  
 535 nm (Mansuy, D., Sassi, A., Dansette, P.M. and Plat, M. A new potent inhibitor of lipid  
 peroxidation *in vitro* and *in vivo*, the hepatoprotective drug anisylditholthione. Biochem.  
 Biophys. Res. Comm. 135: 1015-1021, 1986). The reference characteristics for the standards  
 are listed in the table below.

Compound	IC <sub>50</sub> (μM)
N-Propyl gallate	50
α-Tocopherol	280

Mitogen-activated protein kinase (MAP K) cascades participate in a diverse array of cellular programs. Transmission of signals is achieved by sequential phosphorylation and activation of components to a respective cellular activation cascade. The enzyme for this assay ("MAP-K" in Tables 2-5) [CEREP assay number 768-M] is purchased from Biomol (catalog number SE 137 (0.03 ug/point)). The reaction is carried out in 100 μl volume including an incubation buffer consisting of 100 mM Tris/HCl pH 7.4, 20 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 200 μM Na<sub>3</sub>VO<sub>4</sub>, 2 mM DTT, and 0.2 mg/ml BSA. The substrate for the reaction is 3 μM ATP spiked with [<sup>33</sup>P]-ATP 1 μCi/ assay point. The experiment is developed using a Myelin Basic Protein (MBP) coated flashplate (Robbins, D.J. Zhen, E. Owaki, H. Vanderbilt, C.A. Ebert, T.D. and Cobb, M.H. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J. Biol. Chem., 268: 5097-5106, 1993). This plate includes a surface bearing a coating of the MBP substrate. The general protocol involves the following four steps with the control activity listed in the table below:

1. Preincubation of the flashplate with the enzyme but without ATP: 15 minutes at 30 °C
2. Incubation of the flashplate in the presence of ATP for three hours at 30 °C
3. The reaction is terminated by withdrawal and the plate is washed twice with PBS buffer
4. The overall reaction is determined by measuring the amount of [<sup>33</sup>P] MBP produced using a Topcount (Packard) counter without scintillant. The reference characteristics for the reference standard is listed in the table below.

Compound	IC <sub>50</sub> (μM)
Staurosporine	8.8

The oxytocin receptor binding assay ("Oxytocin" in Tables 2-5) [CEREP assay number 829b] measures binding of [<sup>3</sup>H]-oxytocin to receptors isolated from rat uterus. The reaction is carried out for 1 hour at 22 °C in an incubation buffer consisting of a modified 50 mM Tris-HCL (pH 7.4) buffer. Non-specific binding is estimated in the presence of 1 μM oxytocin. The receptors are collected on membranes by filtration and washed 3 times with ice-cold buffer by vacuum filtration. The washed filters are counted to determine specifically bound [<sup>3</sup>H]-oxytocin (Pettibone, D.J. Identification of functional oxytocin receptors in lactating rat mammary gland in vitro. Eur. J. Pharmacol. 188: 235-241, 1990). The reference characteristics for the reference standards are listed in the table below.

Compound	IC <sub>50</sub> (nM)
Oxytocin	1.6 ± 0.9 (n = 31)
AVP ((Arg-8)-vasopressin)	1.6
[d(CH <sub>2</sub> ) <sub>5</sub> <sup>1</sup> , Tyr(Me) <sub>2</sub> ]AVP	73

The lck proto-oncogene is a member of the *src*-related gene family of non-receptor tyrosine kinases. It is uniquely expressed only in T-lymphocytes and NK-cells. The p56<sup>lck</sup> kinase is a 56 kDa polypeptide that is associated with a cytoplasmic face of the plasma membrane. It functions as a critical signaling model in IL-2 signal transduction leading to T-lymphocyte activation. Specifically, it has been shown that a stable complex exists between IL-2 receptors and p56<sup>lck</sup> and that treatment of T-lymphocytes with IL-2 promotes p56<sup>lck</sup> kinase activity and rapid phosphorylation of cellular substrates. In addition, p56<sup>lck</sup> has been found to function as a signal transducer for antigen-activated CD4 and CD8 T-cell receptors and plays a role in thymic maturation of certain T-cells. The CD45 antigen, a

receptor/protein tyrosine phosphatase of T-cells, also interacts with p56<sup>lck</sup>. The p56<sup>lck</sup> tyrosine kinase is purified from bovine thymus ("p56 lck TK" in Tables 2-5) [Panlabs assay number 17600]. Either test compound and/or vehicle is pre-equilibrated with the enzyme and immobilized synthetic polypeptide (polyGlu:Tyr 4:1) as substrate for 15 minutes. Following a 10-minute kinase reaction in the presence of 100  $\mu$ M ATP, phosphorylated tyrosine residues resulting from activity at the 69 kD kinase domain are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin-linked  $\beta$ -galactosidase enzyme. Fluorescence resulting from conversion of fluorescein-di- $\beta$ -galactoside to fluorescein is measured. The reaction with fluorescein-di- $\beta$ -galactoside is stopped by addition of phenylethyl- $\beta$ -D-thiogalactoside, a reversible competitive inhibitor of  $\beta$ -galactosidase (Cheng, H.C., Nishio, H., Hatase, O., Ralph, S. and Wang, J.H. A synthetic peptide derived from p34cdc2 is a specific and efficient substrate of *src*-family tyrosine kinases. J. Biol. Chem. 267: 9248-9256, 1992). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> ( M)
Staurosporine	0.014
Tyrphostin 47	2.0
Tyrphostin 51	1.8

p59<sup>fyn</sup> is also a member of the *src*-related gene family of non-receptor tyrosine kinases. Recent studies indicate that p59<sup>fyn</sup> tyrosine kinase plays a critical role in mediating signal transduction through the T-cell receptor thereby initiating a signal transduction cascade leading to lymphokine secretion and cell proliferation. One of the earliest biochemical responses following T-cell receptor activation is an increase in tyrosine kinase activity and

concomitant phosphorylation of several intracellular substrates. P59<sup>fyn</sup> tyrosine kinase is an important mediator of this phosphorylation since it is physically associated with the T-cell receptor. Additional evidence supports the view that fyn participates in signal transduction pathways leading from multiple T-cell membrane proteins and hence may serve to integrate stimuli received from several independent receptor structures. In the case of the B-cell receptor, fyn is one of several kinases associated with the receptor and is activated following receptor cross-linking. The p59<sup>fyn</sup> tyrosine kinase is partially purified from bovine thymus for use in this assay ("p59 fyn TK" in Tables 2-5) [Panlabs assay number 17200]. Test compound and/or vehicle is pre-equilibrated with the enzyme and immobilized synthetic polypeptide (polyGlu:Tyr 4:1) as substrate for 15 minutes. Following a 10-minute kinase reaction in the presence of 100  $\mu$ M ATP, phosphorylated tyrosine residues resulting from activity at the 69 kD kinase domain are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin-linked  $\beta$ -galactosidase enzyme. Fluorescence resulting from conversion of fluorescein-di- $\beta$ -galactoside to fluorescein is measured. The reaction with fluorescein-di- $\beta$ -galactoside is stopped by addition of phenylethyl- $\beta$ -D-thiogalactoside, a reversible competitive inhibitor of  $\beta$ -galactosidase (Appleby, M.W. *et al.* Defective T cell receptor signaling in mice lacking the thymic isoform of p59<sup>fyn</sup>. *Cell* 70: 751-763, 1992). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> (μM)
Staurosporine	0.081
Tyrphostin 47	3.2
Tyrphostin 51	11.0

The platelet activating factor (PAF) assay ("PAF receptor" in Tables 2-5) [Panlabs assay number 26500] measures binding of [<sup>3</sup>H]-PAF to PAF receptors. Platelets of male or female New Zealand derived albino rabbits weighing 2.5-3 kg are prepared in modified Tris-HCl pH 7.5 buffer using standard techniques. A 1 mg aliquot of platelet membrane is incubated with 0.4 nM [<sup>3</sup>H]-PAF for 60 minutes at 25 °C. Non-specific binding is estimated in the presence of 1 μM PAF. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [<sup>3</sup>H]-PAF (Hwang, S.B. *et al.* Specific receptor sites for 1-O-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and guinea pig smooth muscle membranes. *Biochemistry* 22: 4756-4763, 1983). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
PAF	9	5.8	1.0
3-(4-[2-chlorophenyl]-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-3,3-a)[1,4]-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone (WEB-2086)	110	71	0.8

Cyclic AMP exerts its effects in animal cells mainly by activating the enzyme cyclic AMP-dependent protein kinase A (PKA) ("PK A, NS" in Tables 2-5) [Panlabs assay number 17700]. This enzyme catalyzes transfer of the terminal phosphate group from ATP to specific serines and threonines of selected proteins. Covalent phosphorylation of the appropriate amino acids of specific cellular proteins in turn results in altered cellular function as a consequence of activation or inactivation of enzymes or altered membrane permeability



to ions or other possible effects. PKA is found in all animal cells, and it is known that the activities of hormones or transmitters may be mediated by cAMP-stimulated phosphorylation of specific sites on neuronal or non-neuronal cells to exert their physiological function. A specific inhibitor of this enzyme should have potential in bioscience research or as a cancer therapeutic. The protein kinase A used for this assay was partially purified from bovine heart (Sigma, P5511). Either test compound and/or vehicle is incubated with the enzyme, [ $\gamma$ - $^{32}\text{P}$ ]-ATP, histone H1, 10 mM  $\text{MgCl}_2$  and 0.1mM  $\text{CaCl}_2$  at 25 °C. The reaction is terminated after 15 minutes by cooling on ice. A 25  $\mu\text{l}$  aliquot is removed and spotted on phosphocellulose paper, washed three times in cold phosphoric acid, dried and counted to determine the amount of  $^{32}\text{P}$ -phosphorylated histone H1 formed (Peters, K.A., Demaille, J.G. and Fischer, E.H. Adenosine 3',5'-monophosphate dependent protein kinase from bovine heart: characterization of the catalytic subunit. *Biochemistry* 16: 5691-5697, 1977). The reference characteristics for the standards are listed in the table below.

Compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )
H-7 [1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine]	3.6
Staurosporine	0.005

The progesterone binding assay ("progesterone" in Tables 2-5) [CEREP assay number 814h] measures binding of [ $^3\text{H}$ ]-R5020 (promogesterone) to human progesterone receptors partially purified from MCF-7 cells (500  $\mu\text{g}$  protein/reaction). The receptors were incubated in a buffer consisting of 5 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  (pH 7.4), 10 mM monothioglycerol, 10% glycerol and 20 mM  $\text{Na}_2\text{MoO}_4$ . The receptors are incubated with 2 nM [ $^3\text{H}$ ]-R5020 for 20 hours at 4 °C. Non-specific binding is estimated in the presence of 1000 nM R5020. Free [ $^3\text{H}$ ]-R5020 is separated from bound radioligand by adsorption of the free radioligand to

dextran-coated charcoal for 10 minutes at 4 °C, followed by pelleting at 3,500 x g for 10 minutes. The supernatants are collected and transferred into vials containing 4 ml of scintillation cocktail (Formula 989, Packard) and counted in a LS 6000 or LS 1701 scintillation counter (Beckman) (Eckert, R.L. and Katzenellenbogen, B. Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. Cancer Res., 42 : 139-144, 1982). The reference characteristics for the standards are listed in the table below.

Compound	IC <sub>50</sub> (μM)
R5020 (promegestone)	21.7 ± 1.6 (n=34)
Progesterone	60
Mibolerone	65
17-β-Estradiol	3,900

The evidence that reactive species of oxygen ( $O_2^-$ ,  $H_2O_2$  and  $OH^\cdot$ ) play an important role in pulmonary oxygen toxicity, inflammation, ischemia/reperfusion injury, etc., and that superoxide dismutase (SOD, EC.1.15.1.1) provides a defense against superoxide radicals, increases continuously. This enzyme catalyzes the dismutation of two superoxide radicals ( $O_2^-$ ) into  $O_2$  and  $H_2O_2$ . Many methods have been devised to measure superoxide dismutase activity; the method described here ("SOD" in Tables 2-5) [Panlabs assay number 19350] is based on the inhibition of nitroblue tetrazolium reduction with xanthine, using xanthine oxidase as a superoxide generator. Agents which decrease superoxide  $O_2^-$  formation may have therapeutic potential for treatment of the above-noted disease states. The enzymes superoxide dismutase (Sigma S-9636, isolated from human erythrocytes) and xanthine oxidase (Sigma X-1875) are used. The test compound and/or vehicle is incubated with SOD, 0.3 mM xanthine as substrate, 0.006 U xanthine oxidase, 150 μM nitroblue tetrazolium, 0.6

mM EDTA, 1% bovine serum albumin and  $\text{Na}_2\text{CO}_3$  at pH 10.2 for 20 minutes at 25 °C.

Conversion of xanthine to formazan is then determined by measurement of absorbance at 540 nm and percent inhibition by superoxide or test compound is calculated (Sun, Y., Oberly, L.W. and Li, Y. A simple method for clinical assay of superoxide dismutase. Clin. Chem.

34: 497-500, 1988). The reference characteristic for the standard is listed in the table below.

Compound	$\text{IC}_{50}$ ( $\mu\text{M}$ )
Superoxide Dismutase (SOD)	0.0021

The testosterone binding assay ("testosterone" in Tables 2-5) [Panlabs assay number 28500] measures binding of [ $^3\text{H}$ ]-mibolerone to testosterone (androgen) receptors. Cytosol from ventral prostate of male Wistar derived rats weighing  $175 \pm 25$  g is prepared in modified phosphate pH 7.2 buffer containing protease inhibitors and triamcinolone acetonide (5  $\mu\text{M}$ ) using standard techniques. A 400  $\mu\text{g}$  aliquot of cytosol protein is incubated with 2 nM [ $^3\text{H}$ ]-mibolerone for 18 hours at 4 °C. Non-specific binding is estimated in the presence of 2  $\mu\text{M}$  mibolerone. The reaction mixture is incubated with a hydroxyapatite slurry for 15 minutes and filtered. The filters are washed 3 times and counted to determine specifically bound [ $^3\text{H}$ ]-mibolerone (Schilling, K. and Shutsung, L. The use of radioactive  $7\alpha$ ,  $17\alpha$ -dimethyl-19-nortestosterone (mibolerone) in the assay of androgen receptors. The Prostate 5: 581-588, 1984). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	0.43 nM
$B_{\text{max}}$	27 fmol/mg protein
Specific Binding	70%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
Cortisol	>10,000	-	-
5- $\alpha$ -Dihydrotestosterone	3.9	0.69	0.9
17- $\beta$ -Estradiol	250	45	1.0
Mibolerone	4.2	0.75	1.1
Testosterone	7.8	1.4	1.0

To measure thromboxane A<sub>2</sub>-induced platelet aggregation ("thromboxane A<sub>2</sub> platelet aggregation" in Tables 2-5), venous blood obtained from male or female New Zealand derived albino rabbits weighing 2.5-3 kg is mixed with one-tenth volume of trisodium citrate (0.13 M) and centrifuged at room temperature for 10 min at 220 x g (Panlabs assay number 47851). Inhibition of platelet aggregation in the supernatant platelet-rich plasma at 37 °C is measured by an optical aggregometer [Panlabs assay number 47851]. Test substances which induce platelet aggregation by more than 50% within 5 min, relative to control 1.5  $\mu$ M thromboxane A<sub>2</sub> mimetic U-46619 response, indicate significant agonist activity. Test substances with the ability to inhibit U-46619-induced platelet aggregation by more than 50% are thromboxane A<sub>2</sub> receptor antagonists (Patscheke, H. and Stregmeier, K. Investigations on a selective non-prostanoid thromboxane antagonist, BM 13,177, in human platelets.

Thrombosis Research 33: 277-288, 1984). The reference characteristic for the standards are listed in the table below.

Compound	IC <sub>50</sub> ( $\mu$ M)
BM-13,505 (Daltroban)	0.34
BM 13,177 (Sultorban)	3.2
Tiaramide	3.5

The tumor necrosis factor (TNF) non-selective assay ("TNF" in Tables 2-5) [Panlabs assay number 28651] measures binding of [ $^{125}$ I]-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) to human TNF- $\alpha$  receptors. U-937 (human histiocytic lymphoma) cells are used to prepare membranes in modified Tris HCl buffer at pH 7.4 using standard techniques. A 200  $\mu$ g aliquot is

5 incubated with 28 pM [ $^{125}$ I]-TNF- $\alpha$  for 3 hours at 4 °C. Non-specific binding is estimated in the presence of 40 nM TNF- $\alpha$ . Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [ $^{125}$ I]-TNF- $\alpha$  (Baglioni, C., McCandless, S., Tavernier, J. and Fiers, W. Binding of human tumor necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. J. Biol. Chem. 260: 13395-13397, 1985). The reference characteristics for the components of the assay and its reference

10 standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	70 pM
$B_{max}$	200 Receptors/cell
Specific Binding	60%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
TNF- $\alpha$	0.12	0.7	1.2
TNF- $\beta$	1.0	0.5	1.0

15 Xanthine oxidase (XOD) catalyzes the hydroxylation of many purine substrates and, in mammals, converts hypoxanthine to xanthine and then uric acid and O<sub>2</sub><sup>-</sup>. The enzyme is widely distributed in mammals, and the abnormal product of uric acid, which often causes gout, remains an issue of concern. In addition, the superoxide anion radical (O<sub>2</sub><sup>-</sup>), generated upon the formation of uric acid, has been found to cause tissue damage. Inhibitors of this

20 enzyme result in increased excretion of purines and should be useful in reducing

hyperurecemia which generally leads to gout. XOD obtained from bovine buttermilk is used in this assay ("Xanthine oxidase" in Tables 2-5) [Panlabs assay number 19800]. The test compound and/or vehicle is pre-incubated with 0.03 U XOD in phosphate buffer pH 7.5 for 10 minutes at 37 °C. The reaction is initiated by addition of 0.5 mM xanthine and run for an additional 30 minutes. The reaction is then terminated by addition of 1 N HCl and absorbance of the mixture at 290 nm is measured. The concentration of uric acid formed is calculated by subtraction of absorbance for the blank solution similarly prepared but with addition of XOD after HCl (Hatano, T., Yasuhara, T., Yoshihara, R., Agata, I., Noro, T. And Okuda, T. Effects of interaction of tannins with co-existing substances VII. Inhibitory effects of tannins and related polyphenols on xanthine oxidase. Chem. Pharm. Bull. 38: 1224-1229, 1990). The reference characteristic for the standard is listed in the table below.

Compound	IC <sub>50</sub> (μM)
Allopurinol	0.98

The estrogen receptor  $\alpha$  binding assay (ER $\alpha$ ) [Panlabs assay number 22600] measures binding of [<sup>3</sup>H]estradiol to estrogen receptors. Cytosol from frozen calf uterus is prepared in modified Tris-HCl pH 7.4 buffer using standard techniques. A 100 μg aliquot of cytosol protein is incubated with 1.5 nM [<sup>3</sup>H]estradiol for 14-16 hours at 4 °C. Non-specific binding is estimated in the presence of 5.8 μM diethylstilbestrol. Bound [<sup>3</sup>H]estradiol is separated from free radioligand by adsorption to dextran-coated charcoal. After low speed centrifugation an aliquot is removed from the supernatant and counted to determine specifically bound [<sup>3</sup>H]estradiol (McGuire, W.L. *et al.* eds. Steroid in Human Breast Cancer. Cancer Res. 38: 4289-4291, 1978). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
$K_d$	0.06 nM
$B_{max}$	42 fmol/mg protein
Specific Binding	75%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
Diethylstilbestrol	0.65	0.025	1.0
17- $\alpha$ -estradiol	1.1	0.041	0.7
Ethinylestradiol	0.081	0.003	1.1

5 The human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptor binding assay [Panlabs assay number 21960] measures binding of [<sup>3</sup>H]spiperone to human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptors. CHO cells stably transfected with a plasmid encoding the human dopamine D<sub>2L</sub> receptor are used to prepare membranes in modified Tris-HCl pH 7.4 buffer using standard techniques. A 20  $\mu$ g aliquot of membrane is incubated with 2 nM [<sup>3</sup>H]spiperone for 120 minutes at 25 °C. Non-specific binding is estimated in the presence of 10  $\mu$ M haloperidol. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [<sup>3</sup>H]spiperone (Bunzo, J.R. *et al.* Cloning and expression of rat D<sub>2</sub> dopamine receptor cDNA. *Nature* **336**: 783-787, 1988; Grandy, D.K. *et al.* Cloning of the cDNA and gene for a human D<sub>2</sub> dopamine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 9762-9766, 1989 and Hayes, G. *et al.* Structural subtypes of the dopamine D<sub>2</sub> receptor are functionally distinct: Expression of the clone D<sub>2A</sub> and D<sub>2B</sub> subtypes in a heterologous cell line. *Mol. Endocrin.* **6**: 920-926, 1992). The reference characteristics for the components of the assay and its reference standards are listed in the two tables below.

Assay Parameter	Result
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K <sub>d</sub>	0.08 nM
B <sub>max</sub>	0.48 pmol/mg protein
Specific Binding	85%

Compound	IC <sub>50</sub> (nM)	K <sub>i</sub> (nM)	nH
R-(-)-Apomorphine	5,600	220	0.8
(+)-Butaclamol	3.4	0.13	0.6
Chlorpromazine	160	6	0.8
Clozapine	7,600	290	0.8
Dopamine	>10,000	-	-
<i>cis</i> -Flupenthixol	0.66	0.025	0.7
Haloperidol	52	2	1.0
SCH-23390	>10,000	-	-
SKF-38393	>10,000	-	-
Spiperone	0.61	0.023	0.7
S-(-)-Sulpiride	1,000	40	0.7

SCH-23390=7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1*H*-3-benzazepine-7-ol

SKF-38393=2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine HCl.

5

The constitutive nitric oxide synthase enzyme expressed in the brain (NOS<sub>c</sub>) is a member of a growing family of enzymes that are known to catalyze the conversion of L-arginine to citrulline and nitric oxide. Like other NOS isoforms, NOS<sub>c</sub> requires NADPH, FAD and tetrahydrobiopterin, as well as calcium/calmodulin. In this assay [Panlabs assay number 14200], the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline is measured by solid phase separation of the compounds and scintillation counting. NOS<sub>c</sub> obtained from a crude rat cerebella cytosol preparation is used. In micrtotitre test plates containing buffer, the compound and/or vehicle, the NOS<sub>c</sub> enzyme and [<sup>3</sup>H]arginine are incubated. The reaction is run for 10 minutes at room temperature. The reaction is terminated by addition of EGTA.

15 Reaction products are separated by ion-exchange chromatography and [<sup>3</sup>H]citrullin is quantitated by scintillation counting (Lowenstein, C.J. and Snyder, S.H. Nitric oxide, a novel biologic messenger. *Cell* 70: 705-707, 1992. and Nathan, C. Nitric oxide as a secretory



product of mammalian cells. FASEB J. 6: 3051-3064, 1992). The reference characteristics for the components of the assay and its reference standards are listed in the table below.

Compound	IC <sub>50</sub> (μM)
N <sup>G</sup> -Monomethyl-L-arginine acetate	2.6
N <sup>G</sup> -Nitro-L-arginine	0.89
N <sup>G</sup> -Nitro-L-arginine methyl ester	1.2
Trifluoperazine	28.0

5

The thyrotropin-releasing hormone receptor binding assay [Panlabs assay number 28600] measures binding of [<sup>3</sup>H]methyl-thyrotropin-releasing hormone (Me-TRH) to  
 10 thyrotropin-releasing hormone receptors. Whole brain (except cerebellum) membranes of male Wistar derived rats weighing 175 ± 25 g are prepared in disodium hydrogen phosphate pH 7.4 buffer using standard techniques. A 16 mg aliquot of membrane is incubated with 3 nM [<sup>3</sup>H]Me-TRH for 120 minutes at 0 °C. Non-specific binding is estimated in the presence  
 15 of 10 μM TRH. Membranes are filtered and washed 3 times and the filters are counted to determine specifically bound [<sup>3</sup>H]Me-TRH (Simasko, S.M. and Horita, A. Characterization and distribution of [<sup>3</sup>H](3MeHis<sup>2</sup>) thyrotropin releasing hormone receptors in rat brain. Life Sci. 30: 1793-1799, 1982 and Taylor, R.L. and Burt, D.R. Properties of [<sup>3</sup>H](3MeHis<sup>2</sup>)-TRH binding to apparent TRH receptors in the sheep central nervous system. Brain Res. 218: 207-217, 1981). The reference characteristics for the components of the assay and its reference  
 20 standards are listed in the two tables below.

Assay Parameter	Result
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$K_d$	5.4 nM
$B_{max}$	34 fmol/mg protein
Specific Binding	76%

Compound	$IC_{50}$ (nM)	$K_i$ (nM)	nH
Chlordiazepoxide	360	230	0.5
DN-1417	14,000	9,000	0.8
MeTRH	3.7	2.4	0.7
MK-771	150	94	0.7
TRH	120	79	0.7

DN-1417= $\gamma$ -butyrolactone- $\gamma$ -carbonyl- L-histidyl-L-prolinamide citrate;

MK-771=L-pyro-2-aminadipyl-histidyl-thiazolidine-4-carboxamide

## 5 Results of Bioassay Tests

The results of the bioassay tests are shown in Figures 2-5, and are expressed as percent inhibition of bioactivity in the assay in the absence of added extract or compound. Using 50% or greater inhibition as an arbitrarily set criterion for a positive result, at least one of the four forms of soybean extracts produced a positive result in ten of 30 assays (Table 2).

- 10 These nine assays are HER2 tyrosine kinase (TK); EGF receptor TK; estrogen receptor  $\beta$  binding; mitogen-activated protein (MAP) kinase; p59<sup>lyn</sup> TK; p56<sup>lck</sup> TK; platelet activating factor (PAF) binding; progesterone receptor binding; ER receptor  $\alpha$  binding and superoxide dismutase (SOD) activity. Furthermore, in each of these assays having positive results, at least one reference compound produced greater than 50% inhibition of activity (tested at
- 15 either 0.01 mM, 0.03 mM, 0.1 mM, or 0.3 mM, depending on the solubility characteristics in the specific assay), and therefore represents a constituent lead for standardizing the soybean extract activity. Interestingly, none of the positive results were generated by the soybean extract oil – all of the positive results were caused by the extracts of the solid soybean forms. However, some of the phytosterols had activity in some bioassays, indicating that their
- 20 activities might be masked.

Table 2

Bioassay	Full Fat Flakes			Defatted Flakes - A			Defatted Flakes B			Soy Oil
	A	D	E	A	D	E	A	D	E	
Adrenergic $\alpha_1$ , NS	18	27	13	22	4	9	27	17	10	10
Adrenergic $\alpha_2$ , NS	6	n/a	1	n/a	10	n/a	18	26	n/a	5
Adrenergic $\beta$ , NS	5	15	12	2	n/a	1	17	13	18	7
EGF	n/a	8	n/a	n/a	15	n/a	4	7	0	8
EGF-TK	22	n/a	n/a	56	n/a	n/a	62	n/a	n/a	4
ERK1 S/TK	n/a	25	n/a	n/a	16	3	13	n/a	n/a	n/a
ER $\beta$	58	35	66	59	41	72	74	25	58	n/a
Glucocorticoid	26	4	n/a	5	n/a	9	n/a	20	n/a	10
HER2 TK	77	n/a	1	86	n/a	19	89	6	11	0
HMG-CoA	21	0	20	21	n/a	4	26	0	4	11
K <sup>+</sup> channel (Ca <sup>++</sup> dependent)	9	18	n/a	15	n/a	n/a	3	n/a	n/a	n/a
IL-6	n/a	n/a	n/a	8	n/a	13	0	n/a	4	5
IL-8, NS	6	19	15	13	10	12	14	14	4	21
Lipid Peroxidation	15	7	n/a	23	4	15	28	n/a	n/a	10
MAP-K	n/a	43	35	n/a	55	46	n/a	44	48	14
Oxytocin	0	10	20	7	8	18	7	5	22	6
p59fyn TK	93	n/a	57	78	n/a	31	86	3	42	n/a
p56lck TK	78	n/a	83	86	n/a	80	89	n/a	80	n/a
PAF receptor	63	17	12	60	16	n/a	58	23	9	n/a
Progesterone	1	28	49	6	31	60	10	38	53	15
PK A, NS	13	n/a	n/a	11	4	n/a	17	n/a	n/a	n/a
SOD	75	8	n/a	77	5	2	76	11	17	n/a
Testosterone	n/a	n/a	n/a	3	n/a	n/a	9	n/a	13	n/a
Thromboxane A <sub>2</sub> platelet aggregation	0	41	0	0	0	2	0	0	0	26
TNF	n/a	n/a	n/a	7	n/a	n/a	13	n/a	n/a	5
Xanthine Oxidase	18	n/a	n/a	28	n/a	n/a	9	n/a	n/a	19
Dopamine 2	13	NT	n/a	22	NT	n/a	NT	NT	NT	NT
ER $\alpha$	n/a	25	n/a	80	n/a	56	NT	NT	NT	NT
NOS c	12	n/a	n/a	n/a	n/a	1	NT	NT	NT	NT
Thyrotropin releasing hormone	15	NT	6	27	14	31	18	NT	n/a	NT

n/a = not active

NT = not tested

A = aqueous extract

D = DMSO extract

E = ethanol extract

expressed as % inhibition of bioactivity in absence of added extract

Of particular interest are the activities of the solid soybean extracts against bioassays that are correlated with various types of cancers (Table 2). For example, HER2 TK has been shown to be correlated with an increased risk of breast cancer. The soy full-fat and defatted flakes had significant activity (arbitrarily set at  $\geq 50\%$  inhibition) in the assays for inhibition of HER2 TK activity, as well as p59<sup>lyn</sup> TK activity, and p56<sup>lck</sup> TK activity (Table 2). Aqueous extracts of full-fat flakes, defatted flakes (A), and defatted flakes (B) inhibited HER2 TK activity by 77%, 86%, and 83%, respectively. The ethanol extracts of these flakes contained much lower levels of activity, namely 0%, 19%, and 11%, respectively. Similarly, aqueous and ethanol extracts of full-fat flakes, defatted flakes (A), and defatted flakes (B) significantly inhibited p56<sup>lck</sup> TK activity, and aqueous extracts of full-fat flakes, defatted flakes (A), and defatted flakes (B) inhibited p59<sup>lyn</sup> TK activity. None of the DMSO extracts had  $> 6\%$  activity in any of these assays. HER2 TK inhibition activity has not been previously found in soy extracts.

Isolated soybean constituents that exhibited significant levels of activity (arbitrarily set at  $\geq 50\%$  inhibition) in the assays correlated with various types of cancer included coumestrol, biochanin A, genistein, and L- $\alpha$ -lysophosphatidylcholine (lyso- $\alpha$ -PC; lysolecithin) (Tables 3-5). In particular, coumesterol exhibited inhibitory activity in a number of these bioassays, as well as others (Table 4). Included among these assays are assays for EGF receptor TK, ERK1 serine/threonine kinase, HER2 TK, MAP kinase, p59<sup>lyn</sup> TK, p56<sup>lck</sup> TK, protein kinase A, estrogen receptor  $\alpha$ , and superoxide dismutase (SOD) activity. Also of interest was the activity profile of lysolecithin, which also showed significant activity in the HER2 TK assay, ERK1 serine/threonine kinase assay, p59<sup>lyn</sup> TK assay, p56<sup>lck</sup> TK assay, and MAP kinase assay (Table 5). Lysolecithin and coumestrol have not been previously associated with effects on these bioactivities.

Table 3

Bioassay	Biochanin A	Daidzin	Daidzein	Genistin	Genistein	Glycitein	6-O-Acetyl Daidzin	6-O-Acetyl Genistin	6-O-Acetyl Glycitin
Adrenergic $\alpha 1$ , NS	42	4	31	0	44	0	41	20	14
Adrenergic $\alpha 2$ , NS	26	n/a	11	8	22	8	15	n/a	n/a
Adrenergic $\beta$ , NS	24	5	9	5	15	5	12	23	4
EGF	n/a	8	8	n/a	n/a	n/a	12	n/a	19
EGF-TK	39	4	20	7	43	7	12	9	12
ERK1 S/TK	15	16	n/a	2	n/a	2	n/a	2	20
ER $\beta$	102	102	100	101	101	98	3	7	n/a
Glucocorticoid	n/a	13	18	10	17	10	3	n/a	n/a
HER2 TK	81	34	67	38	81	38	NT	NT	NT
HMG-CoA	31	n/a	39	23	46	23	n/a	9	12
K <sup>+</sup> channel (Ca <sup>++</sup> dependent)	3	n/a	22	n/a	n/a	n/a	14	n/a	22
IL-6	n/a	8	n/a	n/a	13	n/a	9	9	7
IL-8, NS	n/a	5	n/a	9	n/a	9	n/a	n/a	n/a
Lipid Peroxidation	4	9	17	n/a	21	n/a	15	14	n/a
MAP-K	78	n/a	38	n/a	86	n/a	30	n/a	n/a
Oxytocin	30	n/a	n/a	17	37	9	1	91	1
p59 <sup>lck</sup> TK	96	48	86	78	103	84	NT	NT	NT
p56 <sup>lck</sup> TK	86	1	28	19	76	3	NT	NT	NT
PAF receptor	10	11	n/a	61	4	30	22	29	12
Progesterone	62	5	0	0	15	n/a	n/a	46	n/a
PK A, NS	69	0	n/a	8	41	32	34	29	8
SOD	92	13	26	26	91	24	16	n/a	15
Testosterone	19	n/a	n/a	n/a	28	5	NT	NT	NT
Thromboxane A <sub>2</sub> platelet aggregation	18	12	15	42	13	26	3	3	3
TNF	0	4	35	15	23	17	n/a	n/a	14
Xanthine Oxidase	n/a	n/a	n/a	n/a	n/a	14	n/a	n/a	n/a
Dopamine 2	18	10	7	32	33	28	n/a	43	11
ER $\alpha$	89	100	96	96	45	50	9	7	n/a
NOS c	18	n/a	n/a	14	23	n/a	5	V	n/a
Thyrotropin releasing hormone	n/a	12	5	n/a	24	23	34	26	24

n/a = not active

NT = not tested

Table 4

Bioassay	Campesterol	Coumestrol	$\beta$ -Sitostanol	$\beta$ -Sitosterol (natural)	$\beta$ -Sitosterol (synthetic)	Stigmasterol
Adrenergic $\alpha$ 1, NS	n/a	n/a	5	7	5/13	9
Adrenergic $\alpha$ 2, NS	n/a	0	7	7	17/11	15
Adrenergic $\beta$ , NS	16	11	12	12	7/5	7
EGF	n/a	n/a	7	n/a	5/16	3
EGF-TK	8	86	n/a	14	23/30	8
ERK1 S/TK	n/a	58	0	n/a	n/a	n/a
ER $\beta$	22	82	11	n/a	n/a	7
Glucocorticoid	n/a	6	24	n/a	n/a	n/a
HER2 TK	51	89	26	40	40/46	39
HMG-CoA	31	n/a	n/a	1	n/a	14
K <sup>+</sup> channel (Ca <sup>++</sup> dependent)	n/a	n/a	n/a	n/a	n/a	n/a
IL-6	15	n/a	1	n/a	n/a	n/a
IL-8, NS	0	n/a	1	5	n/a	n/a
Lipid Peroxidation	n/a	45	24	9	2/1	24
MAP-K	21	54	n/a	n/a	n/a	22
Oxytocin	6	n/a	22	14	15	13
p59 <sup>lck</sup> TK	96	100	20	30	58/71	60
p56 <sup>lck</sup> TK	91	97	n/a	n/a	n/a	n/a
PAF receptor	n/a	46	n/a	n/a	n/a	n/a
Progesterone	n/a	20	n/a	n/a	n/a	n/a
PK A, NS	n/a	93	n/a	4	5/12	n/a
SOD	n/a	125	1	n/a	n/a	n/a
Testosterone	n/a	13	n/a	n/a	n/a	4
Thromboxane A <sub>2</sub> platelet aggregation	20	27	6	19	19/13	0
TNF	16	n/a	n/a	29	n/a	10
Xanthine Oxidase	16	n/a	1	25	n/a	18
Dopamine 2	n/a	18	n/a	NT	10	n/a
ER $\alpha$	22	10	28	NT	30	6
NOS c	n/a	10	19	NT	8	n/a
Thyrotropin releasing hormone	8	26	26	NT	10	5

n/a = not active

NT = not tested

Table 5

Bioassay	L- $\alpha$ -lyso-PC	L- $\alpha$ -PC	L- $\alpha$ -PE	L- $\alpha$ -PI	L- $\alpha$ -PS	Raffinose	Stachyose	Tocopherol	Trypsin Inhibitor
Adrenergic $\alpha_1$ , NS	56	7	1	18	n/a	6	13	12	1
Adrenergic $\alpha_2$ , NS	69	12	0	19	n/a	1	1	n/a	n/a
Adrenergic $\beta$ , NS	41	10	27	n/a	n/a	26	13	7	21
EGF	100	n/a	n/a	7	n/a	0	n/a	4	6
EGF-TK	41	45	8	n/a	30	26	n/a	26	n/a
ERK1 S/TK	65	n/a	n/a	80	31	7	12	2	n/a
ER $\beta$	48	n/a	5	65	26	n/a	2	n/a	21
Glucocorticoid	86	n/a	n/a	n/a	n/a	n/a	7	8	n/a
HER2 TK	74	39	62	15	n/a	n/a	n/a	69	48
HMG-CoA	34	32	32	25	37	24	27	13	28
K <sup>+</sup> channel (Ca <sup>++</sup> dependent)	58	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
IL-6	11	n/a	n/a	2	n/a	7	n/a	n/a	15
IL-8, NS	103	13	21	14	19	n/a	5	11	4
Lipid Peroxidation	n/a	3	8	n/a	3	20	15	19	10
MAP-K	112	n/a	n/a	7	n/a	n/a	9	n/a	94
Oxytocin	43	7	3	n/a	0	1	n/a	n/a	22
p59 <sup>lck</sup> TK	111	109	68	90	80	14	28	78	28
p56 <sup>lck</sup> TK	97	93	92	92	91	6	12	16	n/a
PAF receptor	88	20	5	n/a	4	3	n/a	4	n/a
Progesterone	4	5	n/a	10	3	3	4	20	1
PK A, NS	69	n/a	n/a	n/a	n/a	23	n/a	50	24
SOD	n/a	6	n/a	n/a	3	6	14	23	n/a
Testosterone	25	n/a	n/a	n/a	n/a	12	17	24	0
Thromboxane A <sub>2</sub> platelet aggregation	36	0	0	0	1	4	4	0	0
TNF	70	33	28	14	24	n/a	24	13	21
Xanthine Oxidase	47	9	n/a	19	33	14	21	n/a	14
Dopamine 2	87	n/a	n/a	n/a	n/a	19	2	n/a	11
ER $\alpha$	4	n/a	n/a	61	NT	18	8	20	66
NOS c	15	n/a	n/a	n/a	NT	n/a	n/a	17	n/a
Thyrotropin releasing hormone	27	20	n/a	4	NT	8	28	11	9

n/a = not active

NT = not tested

L- $\alpha$ -lysoPC = L- $\alpha$ -lysophosphatidylcholine; L- $\alpha$ -PC = L- $\alpha$ -phosphatidylcholine;5 L- $\alpha$ -PE = L- $\alpha$ -phosphatidylethanolamine; L- $\alpha$ -PI = L- $\alpha$ -phosphatidylinositol; L- $\alpha$ -PS = L- $\alpha$ -phosphatidylserine

The soybean extracts also exhibited significant activity in the estrogen receptor  $\beta$

(Erb) assay, which is correlated with osteoporosis. For example, the aqueous extracts of full-

fat and defatted flakes show significant levels of activity in the ER  $\beta$  assay. Aqueous and

10 ethanol extracts of full-fat flakes (A) exhibited 58% and 66% inhibition of ligand binding to

ER  $\beta$ , respectively, while the DMSO extract exhibited only 35% inhibition. Similarly, in the

cases of defatted flakes, the ethanol-extracted samples exhibited 58% and 72% inhibition of ER  $\beta$  ligand binding, respectively, and the aqueous extracts exhibited 74% and 59% inhibition, respectively. In contrast, the DMSO extracts exhibited 25% and 41% inhibition of ER  $\beta$  ligand binding, respectively. Isolated soybean constituents that exhibited activity in the Erb assay included daidzein (110%), genistein (104%), daidzin (102%), coumestrol (82%), genistin (93%), glycitein (93%), and L- $\alpha$ -phosphatidyl inositol (65%) (Tables 3-5).

In addition to the ER  $\beta$  assay, the p59<sup>fyn</sup> TK assay is also associated with osteoporosis. As noted above, aqueous extracts of full-fat flakes, defatted flakes (A), and defatted flakes (B) inhibited p59<sup>fyn</sup> TK activity, and the phospholipids significantly inhibited this activity.

With the exception of the PAF receptor binding inhibition assay, none of the soy extracts exhibited significant activity in assays correlated with cardiovascular disorders. In addition to the PAF receptor binding inhibition assay, these assays included the adrenergic  $\alpha_1$  non-selective assay, the adrenergic  $\alpha_2$  non-selective assay, the adrenergic  $\beta$  non-selective assay, the HMG-CoA reductase assay, the K<sup>+</sup> channel (Ca<sup>++</sup>-dependent) assay, the oxytocin receptor binding inhibition assay, lipid peroxidation, and the thromboxane A<sub>2</sub> platelet aggregation assay. Aqueous extracts of the full-fat flakes, defatted flakes (A), and defatted flakes (B) all exhibited significant levels of activity in the PAF receptor binding inhibition assay. However, a component of soy extracts, lysophosphatidylcholine (lysolecithin) exhibited significant activity in three of these seven assays (Table 5). Additionally, the isoflavone genistin (but not its related aglycone compound genistein) exhibited a significant level of activity in the PAF-R assay. The low levels of bioactivity in these assays by the various soy extracts may represent instances of masking of the bioactivities of lysolecithin and/or genistin. This result highlights the need for multiple bioassays of functional food



products, in order to obtain a standardized functional food product that exhibits the maximal beneficial health effect.

The remaining assays address other potential bioactivities that may be associated with soy. For example, the glucocorticoid receptor binding inhibition assay, the IL-6 receptor binding inhibition assay, and the IL-8 receptor binding inhibition assay all are correlated with anti-inflammatory bioactivity. None of the various soy extracts exhibited significant (arbitrarily set at  $\geq 50\%$  inhibition) levels of activity in these assays. The only compound that showed significant levels of bioactivity in these assays was lysolecithin, which exhibited significant levels of activity in the glucocorticoid receptor binding inhibition assay and the IL-8 receptor binding inhibition assay. As noted above, this may represent an example of masking in these bioassays. The lipid peroxidation assay and the xanthine oxidase assay are reportedly associated with anti-oxidation, and also perhaps anti-aging, bioactivity. Again, none of the various soy extracts exhibited significant levels of activity in these bioassays. The only compound showing significant levels of bioactivity in these assays is soybean trypsin inhibitor, which had activity in the lipid peroxidation assay.

Activity in the progesterone receptor binding inhibition assay and the testosterone receptor binding inhibition assay may be related to the reported antiestrogenic effects of soy. Ethanolic extracts of soy defatted flakes exhibited significant levels of activity in only the progesterone assay, and not in the testosterone assay. Ethanolic extracts of full-fat flakes (A), at 49% inhibition of the progesterone assay, was very nearly a significant inhibitor in this assay. However, the ethanolic extracts of both defatted flake samples had significant activity in the progesterone assay. Of the compounds tested, only the metabolite biochanin A exhibited a significant level of bioactivity, and only in the progesterone assay. The lack of significant levels of activity, particularly with respect to the isoflavones daidzein and

genistein, which have been reported to have antiestrogenic activity in animals and humans, may reflect the presence of synergistic interactions involving these components. It is interesting to note that isoflavones are found principally in the full fat and defatted flake fractions, where significant levels of bioactivity in the progesterone assay were found.

5 Oxytocin binding is associated with premenstrual tension syndrome. Only the ethanol extracts of soy flakes had any activity in this assay (Table 2), and these levels were modest. The most active constituent is the phospholipid L- $\alpha$ -lyso PC (Table 5), followed by the isoflavone genistein (Table 3).

10 The bioactivities ER  $\beta$  binding and adrenergic  $\alpha$ 2 receptor binding are associated with postmenopausal syndrome. As noted above for osteoporosis, the aqueous extracts of full-fat and defatted flakes show significant levels of activity in the ER  $\beta$  assay. Isolated soybean constituents that exhibited activity in the ER  $\beta$  assay were daidzein, genistein, daidzin, coumestrol, genistin, glycitein, and L- $\alpha$ -phosphatidyl inositol (Tables 3-5). None of the extracts showed high levels of activity in the adrenergic  $\alpha$ 2 receptor binding assay, and the  
15 only constituent that showed significant levels of bioactivity was L- $\alpha$ -lyso PC (Table 5).

A more complex picture of bioactivity of the various soy extracts, as well as the isolated soy constituents, is obtained when the minimal level of significant activity is lowered to 20%. For example, significant levels of activity in various bioassays are now detected in the DMSO extracts of full-fat flakes (A). However, some of these same bioactivities are not  
20 at significant levels in the defatted flakes. These bioactivities begin to be of greater interest (but remain below significant levels) in the soy oil fractions (Table 2, thromboxane A<sub>2</sub> platelet aggregation assay). In others, the activity remains in the defatted flakes (Table 2, MAP kinase assay; ER  $\beta$  assay). These assays provide indications of bioactivities that may

well be important in ensuring the health benefits of the functional food product, but are not as immediately apparent as those that exhibit strong bioactivities.

#### Preparation of Specific Soy Functional Food Formulations

5           The goal of the PharmaPrint Process is to determine sets of bioactivity and chemical constituent fingerprints that will allow the precise control of the soy formulation. Unlike the standardization methods of the prior art, which almost without exception standardize on one or at most a very few chemical constituents, the methods of the present invention combine the use of bioassays and determinations of the chemical constituents to standardize the soy  
10       formulations. The first step in the preparation of specific soy functional food formulations is to obtain data regarding the bioactivity of various soy preparations, as measured in one or more bioassays. The bioassays may be selected as those measuring bioactivities known or thought to be present in a soy formulation. The bioactivities are tested at a high concentration of each soy formulation, to ensure that the maximal activity is determined.  
15       Then, each bioactivity is measured as a function of the concentration of the soy formulation. Preferably, the bioactivities are expressed as an  $IC_{50}$  or  $EC_{50}$  value in each case. Alternatively, the bioactivities may be expressed as a given activity, i.e., percent inhibition or activation, at a specified concentration. As noted previously, different soy extracts may exhibit widely different bioactivities (see Table 1C).

20           Referring now to Figure 1A, a soy extract was tested at a concentration of 1 mg/ml in a total of 30 bioassays. The bioassays in which one or more of the soy formulations is able to either inhibit or enhance the activity by a designated amount are further considered as a bioassay useful in the standardization of a soy formation. As an example, a bioassay may be required to either inhibit or enhance a bioactivity, as measured in a bioassay, by at least 80%

to be further considered. In the case of the soy extract of Figure 1A, bioassays 8, 9, 11, 20, 21, and 25 would meet this standard, and would be further considered as being potentially useful in the characterization of this soy formulation. However, a bioassay that does not meet this standard may still be further considered if, for example, it is known that a key biological activity measured by the bioassay is affected by a known constituent of soy or the soy formulation. In this case, the soy formulation may be fractionated, using the bioassay as a guide, to identify the fraction containing the active component that may be masked in the unfractionated soy formulation.

The soy extract is next assayed for the presence and concentration of chemical constituents. These chemical constituents assayed may be those that are known or suspected to be active components of the soy extract. Alternatively, the chemical constituents assayed may be those known or suspected to be present in significant quantities in the soy extract. It is important to select those constituents present at sufficient concentration to permit reproducible, robust characterization of the soy extract. Referring to Figure 1B, a total of 24 different constituents of soy full flake or defatted flake were analyzed. As with the bioassays, a certain minimal amount must be present in order to further consider the constituent for inclusion in the soy extract standardization protocol. However, unlike the case in the bioassays, the minimal amount of each constituent may vary, depending on the ability of the assay to detect the component. At this point, it is not obvious which constituents are candidates for use in the standardization protocols - unless the constituents are present in such low quantities as to lack utility as a standardization constituent. However, even if a constituent cannot be detected in the raw soy formulation, if it is considered to be especially useful, i.e., is uniquely correlated with a specific activity of the soy formulation, methods to

isolate or concentrate the constituent may be brought to bear to enhance the utility of the constituent measurement.

The next step is to select the appropriate bioassays and chemical constituents for inclusion in the standardization protocol. The bioassays and chemical constituents should be chosen as those that are correlated with the desired activity. For example, as noted above, breast cancer is associated with HER2 tyrosine kinase activity, and coumestrol may inhibit activities associated with breast cancer. Estrogen receptor  $\beta$  activity is associated with osteoporosis, and daidzein, genistein, and daidzin may inhibit activities associated with osteoporosis.

The following sections disclose examples of soy functional food formulations prepared for various specific conditions. The methods employed in these examples may be used in preparing formulations directed at other conditions.

#### Soy Functional Food Formulation for Osteoporosis Prevention

Figures 2A-2F and 3A-3F present bioassay data obtained for various soy formulations, with respect to bioassays associated with osteoporosis development. Each soy formulation was extracted with either water (2A, 2B, 3A, 3B), DMSO (2C, 2D, 3C, 3D) or ethanol (2E, 2F, 3E, 3F). Each extract then was tested in bioassays selected for correlation with osteoporosis, at a concentration of 1 mg/ml. Thereafter, selected extracts (water or ethanol) and potential bioactive components were tested in bioassays to determine an  $IC_{50}$  concentration (Table 6).

Table 6

Materials	IC <sub>50</sub> (mg/ml)				
	ER $\alpha$	ER $\beta$	EGF Receptor TK	p59 <sup>lyn</sup> TK	p56 <sup>lck</sup> TK
Novasoy 400 (aqueous)	+	0.0012	0.227	0.188	0.074
Novasoy 400 (ethanol)	+	0.0018	0.339	0.261	0.0414
Soylife 150 (aqueous)	+	0.0031	0.22	0.308	0.386
Soylife 150 (ethanol)	+	0.0073	0.617	0.531	0.573
Active Components	IC <sub>50</sub> (mM)				
	ER $\alpha$	ER $\beta$	EGF Receptor TK	p59 <sup>lyn</sup> TK	p56 <sup>lck</sup> TK
Ipriflavone	NT	NT	NT	NT	NT
Daidzein	+	<0.001	-	0.211	-
Dadzin	+	0.001	-	+	-
Genistein	+	<0.001	+	0.245	+
Genistin	+	0.008	-	0.25	-
Glycitein	+	<0.001	-	0.111	-
Glycitin	NT	-	-	+	-
L- $\alpha$ -PI	+	0.167	-	>0.6	+
L- $\alpha$ -PS	NT	-	-	>0.06	+
Stigmasterol	+	-	-	0.101	-
Tocopherol	-	-	-	0.007	-
BBI	+	-	-	-	-
L- $\alpha$ -Lyso PC	-	0.39	+	0.005	+
L- $\alpha$ -PC	-	-	+	0.05	+
L- $\alpha$ -PE	-	-	-	>0.6	+
Campesterol	-	-	-	>0.6	+

+ known to have activity in the bioassay

- no activity in the bioassay

NT = not tested

5 As expected, the soy formulations were most effective in the Erb (ER  $\beta$ ) bioassay, with IC<sub>50</sub> values ranging from 1.2 to 7.3  $\mu$ g/ml. The soy formulations were at least an order of magnitude less inhibitory in the other bioassays tested. However, these bioassays still may be made a part of the standardized soy functional food product fingerprint - despite the fact that the soy formulations are more inhibitory of the ER  $\beta$  assay, one or more of the other

10 assays may be preferred on grounds of specificity, cost, ease of assay, and the like.

The most effective inhibitory compounds in the ER  $\beta$  assay were daidzein, dadzin, genistein, genistin, and glycitein. Each of these compounds had an  $IC_{50}$  concentration below 10  $\mu$ M, and daidzein, dadzin, genistein, and glycitein had an  $IC_{50}$  concentration at or below 1  $\mu$ M. In addition, it is preferable to include ipriflavone, since there is a great deal of information indicating this compound provides beneficial effects in the treatment or prevention of osteoporosis.

#### Soy Functional Food Formulation for Cardiovascular Health

Figures 2A, 2C, 2E; 4A-4B; 5A-5F; 6A-6E; and 7A-7F present bioassay data obtained for various soy formulations, with respect to bioassays associated with cardiovascular health and the prevention or treatment of heart disease. Each soy formulation was extracted with either water (2A, 4A, 5A, 5B, 6A, 6B, 7A, 7B), DMSO (2C, 5C, 5D, 6C, 7C, 7D) or ethanol (2E, 4B, 5E, 5F, 6E, 6F, 7E, 7F). Each extract then was tested in bioassays selected for correlation with cardiovascular conditions, at a concentration of 1mg/ml. Thereafter, selected extracts (water or ethanol) and potential bioactive components were tested in bioassays to determine an  $IC_{50}$  concentration (Table 7).

Table 7

Materials	IC <sub>50</sub> (mg/ml)							
	HMG CoA reductase	Lipid Peroxidation	PAF-R Binding	SOD	Thromboxane A <sub>2</sub>	ER $\alpha$	ER $\beta$	NOSc
Novasoy 400 (aqueous)	0.0337	-	-	0.302	1.2	+	0.0012	+
Novasoy 400 (ethanol)	0.0089	>10	-	0.374	1.3	+	0.0018	
Soylife 150 (aqueous)	0.0551	-	-	2.34	1.5	+	0.0031	
Soylife 150 (ethanol)	0.103	-	-	2.43	1.9	+	0.0073	+
Centroflo 8215	+ <sup>1,2</sup>	-	-	-	-	-	+ <sup>2</sup>	-
Supro FXP H1061	+ <sup>2</sup>	-	0.18 <sup>1</sup>	-	±	+ <sup>2</sup>	+ <sup>1,2</sup>	-
Active Components	IC <sub>50</sub> (mM)							
Genistein	+	-	0.495	+	-	+	<0.001	-
Daidzein	+	-	-	-	-	+	<0.001	-
L- $\alpha$ -Lyso PC	+	-	0.131	-	+	-	0.39	-
L- $\alpha$ -PS	+	-	-	-	-	-	-	-
L- $\alpha$ -PC	+	-	-	-	-	-	-	-
L- $\alpha$ -PE	+	-	-	-	-	-	-	-
BBI	-	+	-	-	-	+	-	-
Genistin	-	-	-	+	+	-	0.008	-
L- $\alpha$ -PI	-	-	-	-	-	+	0.167	-
Stigmasterol	-	-	-	-	-	+	-	-
Glycitein	-	-	-	-	-	-	<0.001	-

1 aqueous extract

2 ethanol extract

+ known to have activity in the bioassay

- no activity

5

10

As previously noted, there were no bioassays specific to cardiovascular conditions that were highly sensitive to the soy formulations tested. Besides the ER  $\beta$  bioassay, the soy formulations were most effective in the HMG CoA reductase bioassay, with IC<sub>50</sub> values ranging from 8.9 to 103  $\mu$ g/ml. The soy formulations were roughly an order of magnitude less inhibitory in the other bioassays tested. However, these bioassays still may be made a part of the standardized soy functional food product fingerprint.

The most effective inhibitory compounds in the ER  $\beta$  assay were daidzein, genistein, genistin, and glycitein. Each of these compounds had an IC<sub>50</sub> concentration below 10  $\mu$ M,



and daidzein, genistein, and glycitein had an  $IC_{50}$  concentration at or below 1  $\mu$ M. L- $\alpha$ -Lyso PC and L- $\alpha$ -PI both were also inhibitory in the ER  $\beta$  bioassay, at higher  $IC_{50}$  concentrations. Genistein and L- $\alpha$ -Lyso PC both were also inhibitory in the PAF-receptor binding inhibition bioassay, with  $IC_{50}$  values of 0.131 and 0.495 mM, respectively.

5

#### Soy Functional Food Formulation for Postmenopausal Symptoms

Figures 2A, 2C, 2E; 4A, 4B; 6A, 6C, 6D; 8A-8C; and 11B, 11D, 11F present bioassay data obtained for various soy formulations, with respect to bioassays associated with postmenopausal symptoms. Each soy formulation was extracted with either water (2A, 4A, 6A, 11B), DMSO (2C, 6C, 11D) or ethanol (2E, 4B, 6D, 11F). Each extract then was tested in bioassays selected for correlation with postmenopausal symptoms, at a concentration of 1 mg/ml. Thereafter, selected extracts (water or ethanol) and potential bioactive components were tested in bioassays to determine an  $IC_{50}$  concentration (Table 8).

10

Table 8

Materials	IC <sub>50</sub> (mg/ml)				
	Adrenergic $\alpha$ 2	ER $\alpha$	ER $\beta$	PAF Receptor	Progesterone Receptor
Novasoy 400 (aqueous)	0.686	+	0.0012	-	0.38
Novasoy 400 (ethanol)	0.588	+	0.0018	-	1.4
Soylife 150 (aqueous)	1.22	+	0.0031	-	0.59
Soylife 150 (ethanol)	0.652	+	0.0073	-	0.91
Soyarich I (aqueous)	3.16		0.188	0.48	
Soyarich I (ethanol)	3.79	+	0.0511		+
Supro FXP H1061 (aqueous)	2.27		+	0.18	-
Supro FXP H1061 (ethanol)	3.67	+	+	>3	-
Active Components	IC <sub>50</sub> (mM)				
L- $\alpha$ -Lyso PC	+	-	0.39	0.131	-
Daidzein	-	+	<0.001	-	-
Dadzin	-	+	0.001	-	-
Genistein	-	+	<0.001	0.495	-
Genistin	-	+	0.008	-	-
Glycitein	-	+	<0.001	-	-
Glycitin	-	NT	-	-	-
L- $\alpha$ -PI	-	+	0.167	-	-
L- $\alpha$ -PS	-	NT	-	-	-
Stigmasterol	-	+	-	-	-
BBI	-	+	-	-	-
6-O-Acetyl Genistin	-	-	-	-	+

1 aqueous extract

2 ethanol extract

+ known to have activity in the bioassay

- no activity

5

Most of the extracts demonstrated ER  $\beta$  activity, as previously noted in Tables 6 and 7. In addition, some extracts had high progesterone receptor binding inhibition activity, and this correlated with adrenergic  $\alpha$ 2 receptor binding inhibition activity. Only low levels of ER

$\alpha$  receptor binding activity were detected in extracts, and this followed through into the components tested. PAF receptor binding inhibition activity was seen in only two extracts, and the active components were L- $\alpha$ -Lyso PC and dadzin. None of the tested components exhibited activity in either the adrenergic  $\alpha_2$  receptor binding inhibition assay or the progesterone receptor binding inhibition assay, possibly indicating that masking is occurring in these assays.

#### Soy Functional Food Formulation for Breast Cancer

Figures 2A, 2C, 2F; 4A, 4B; 7B, 7D, 7F; 9A-F, and 10A-C present bioassay data obtained for various soy formulations, with respect to bioassays associated with breast cancer. Each soy formulation was extracted with either water (2A, 4A, 7B, 9A, 10A), DMSO (2C, 7D, 9C, 9D, 10B) or ethanol (2E, 4B, 7F, 9E, 9F, 10C). Each extract then was tested in bioassays selected for correlation with breast cancer, at a concentration of 1mg/ml. Thereafter, selected extracts (water or ethanol) and potential bioactive components were tested in bioassays to determine an  $IC_{50}$  concentration (Table 9).

Table 9

Materials	IC <sub>50</sub> (mg/ml)					
	HER2 TK	MAP Kinase	EGF Receptor TK	ER $\alpha$	ER $\beta$	SOD
Centroflo 8215	+	+	+	-	+	-
Soyarich B	+	+	+	-	-	-
Active Components	IC <sub>50</sub> (mM)					
BBI	0.049	+	-	+	-	-
Daidzein	0.063	-	-	+	<0.001	-
Genistein	0.04	+	+	+	<0.001	+
L- $\alpha$ -Lyso PC	0.181	+	+	-	0.39	-
Tocopherol	0.584	-	-	-	-	-
L- $\alpha$ -PC	-	-	+	-	-	-
Daidzin	-	-	-	+	0.001	-
Genistin	-	-	-	+	0.008	-
Glycitein	-	-	-	+	<0.001	-
L- $\alpha$ -PI	-	-	-	+	0.167	-
Stigmasterol	-	-	-	+	-	-

1 aqueous extract

2 ethanol extract

+ known to have activity in the bioassay

- no activity

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As expected, the soy formulations were active in the HER2 TK, MAP kinase, and EGF receptor TK bioassays, all of which are known to correlated with breast cancer.

However, as noted above and as is true in all cases, the other bioassays still may be made a part of the standardized soy functional food product fingerprint.

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The most effective inhibitory compounds in the HER2 TK assay were daidzein, genistein, and Bowman-Birk trypsin inhibitor. Each of these compounds had an IC<sub>50</sub> concentration below 100  $\mu$ M. Also effective in the HER2 TK assay were L- $\alpha$ -Lyso PC and tocopherol, with IC<sub>50</sub> values < 1 mM. In these formulations, it is preferable to minimize the amounts of estrogen receptor  $\alpha$  binding inhibition activity, estrogen receptor  $\beta$  binding

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inhibition activity, and inhibition of superoxide dismutase activity, since these activities are inversely correlated with a positive influence on breast cancer.

#### Soy Functional Food Formulation for Premenstrual Tension Syndrome

5            Figures 13A-F and 12A-D present bioassay data obtained for various soy formulations, with respect to bioassays associated with cardiovascular health and the prevention or treatment of heart disease. Each soy formulation was extracted with either water (11A, 11B, 12A, 12B), DMSO (11C, 11D) or ethanol (11E, 11F, 12C, 12D). Each extract then was tested in bioassays selected for correlation with premenstrual tension  
10            syndrome, at a concentration of 1mg/ml. Thereafter, selected extracts (water or ethanol) and potential bioactive components were tested in bioassays to determine an IC<sub>50</sub> concentration (Table 10).

Table 10

Materials	IC <sub>50</sub> (mg/ml)			
	Oxytocin Receptor	Progesterone Receptor	Dopamine D <sub>2</sub> S	Thyrotropin Releasing Hormone
Novasoy 400 (aqueous)	0.59	0.38	0.227	+
Novasoy 400 (ethanol)	0.6	1.4	0.339	+
Active Components	IC <sub>50</sub> (mM)			
Genistein	+	-	±	-
L-α-Lyso PC	+	-	+	-
6-O-Acetyl Genistin	+	+	+	-
Genistin	-	-	±	-

15            + known to have activity in the bioassay  
             ± minimal detectable activity  
             - no activity

Both extracts tested exhibited activity in all four assays associated with premenstrual tension syndrome. However, none of the compounds tested had activity in the thyrotropin releasing hormone receptor binding inhibition assay. It is possible that another compound is responsible for the activity in the extracts exhibited in this bioassay. Alternatively,  
5 synergistic interactions may be giving rise to the extract bioactivity.

Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of various aspects of the invention. Thus, it is to be understood that numerous modifications may be made in the illustrative embodiments and other arrangements may be devised without  
10 departing from the spirit and scope of the invention. Throughout this application various publications are cited. The contents of these publications are hereby incorporated by reference into the present application.

CLAIMS

What is claimed is:

1. A method for standardizing a botanical extract to create a standardized  
5 functional food product or a standardized dietary supplement, comprising the steps of:
  - a) removing at least one first aliquot from said extract;
  - b) determining from said at least one first aliquot a bioactivity value for said extract,  
using an extract bioassay to measure said bioactivity value;
  - c) comparing said bioactivity value for said extract to a bioactivity standard that  
10 specifies one of a minimal total bioactivity, a maximal total bioactivity, and a range of total  
bioactivities required for a standardized functional food product or a standardized dietary  
supplement as measured using said extract bioassay to provide a bioactivity comparison;
  - d) removing at least one second aliquot from said extract;
  - e) fractionating said at least one second aliquot into a plurality of fractions, said  
15 plurality of fractions containing one or more active components;
  - f) determining from among said plurality of fractions the amount in said extract of at  
least one of said one or more active components, said at least one of said one or more active  
components having activity in an active component bioassay;
  - g) comparing the amount of said at least one of said one or more active components  
20 determined in step f) to an active component standard for a standardized functional food  
product or a standardized dietary supplement which specifies one of a minimal amount, a  
maximal amount, and a range of amounts of said at least one of said one or more active  
components to provide a quantitative compositional fingerprint comparison; and

(h) determining whether said extract is a standardized functional food product or a standardized dietary supplement based on said bioactivity comparison and said quantitative compositional fingerprint comparison.

5           2.     The method of claim 1, wherein said botanical extract is an extract of soy.

          3.     The method of claim 1, wherein said botanical extract is an extract of a member selected from the group consisting of wheat bran, gelatin, and cranberries.

10          4.     The method of claim 2, wherein said extract of soy is one of soy meal, soy flakes, defatted soy flakes, soy sauce, soybean oil, tofu, soy milk, soy flour, soy nuts, tempeh, miso, and natto.

          5.     The method of claim 1, wherein said extract bioassay is one of adrenergic  $\alpha 1$  receptor binding, NS; adrenergic  $\alpha 2$  receptor binding, NS; adrenergic  $\beta$  receptor binding, NS; human dopamine  $D_{2L}$  ( $D_{2A}$ ) receptor binding; EGF receptor binding; EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2 tyrosine kinase activity; HMG CoA reductase activity;  $Ca^{++}$ -dependent  $K^+$  channel binding; IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity; constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>lyn</sup> tyrosine kinase; p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane  $A_2$ -induced platelet

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aggregation; thyrotropin releasing hormone receptor binding; TNF receptor binding; and xanthine oxidase activity.

6. The method of claim 1, wherein said active component bioassay is one of  
5 adrenergic  $\alpha 1$  receptor binding, NS; adrenergic  $\alpha 2$  receptor binding, NS; adrenergic  $\beta$   
receptor binding, NS; human dopamine  $D_{2L}$  ( $D_{2A}$ ) receptor binding; EGF receptor binding;  
EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen  
receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2  
tyrosine kinase activity; HMG CoA reductase activity;  $Ca^{++}$ -dependent  $K^+$  channel binding;  
10 IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity;  
constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>lck</sup> tyrosine kinase;  
p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A  
activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane  $A_2$ -  
induced platelet aggregation; thyrotropin releasing hormone receptor binding; TNF receptor  
15 binding; and xanthine oxidase activity.

7. The method of claim 1, wherein said active component is one or more of  
daidzein; genistein; glycitein; daidzin; genistin; glycitin; 6-O-acetyl daidzin; 6-O-acetyl  
genistin; 6-O-acetyl glycitin; 6-O-malonyl daidzin; 6-O-malonyl genistin; 6-O-malonyl  
20 glycitin; L- $\alpha$ -lysophosphatidylcholine; L- $\alpha$ -phosphatidylcholine; L- $\alpha$ -  
phosphatidylethanolamine; L- $\alpha$ -phosphatidylinositol; L- $\alpha$ -phosphatidylserine; campesterol;  
 $\beta$ -sitostanol;  $\beta$ -sitosterol; stigmasterol; coumestrol;  $\alpha$ -tocopherol; and Bowman-Birk trypsin  
inhibitor.

8. The method of claim 1, wherein said extract bioassay is the same as said active component bioassay.

9. The method of claim 1, wherein said extract bioassay is different from said active component bioassay.

10. The method of claim 1, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cancer.

11. The method of claim 1, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to osteoporosis.

12. The method of claim 1, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to premenstrual tension syndrome.

13. The method of claim 1, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to postmenopausal symptoms.

14. The method of claim 1, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cardiovascular diseases.

15. The method of claim 1, wherein at least one of said plurality of fractions contains a single essentially purified chemical compound.

16. The method of claim 1, wherein said steps a) through h) are repeated at least once.

17. The method of claim 1, further comprising:

- 5 i) fractionating at least one of said plurality of fractions into a plurality of subfractions, said plurality of subfractions containing one or more active components;
- ii) determining from among said plurality of subfractions the amount in said extract of at least one of said one or more active components, said at least one of said one or more active components having activity in said active component bioassay; and
- 10 iii) comparing the amount of said at least one of said one or more active components determined in step ii) to a standard for a standardized functional food product or a standardized dietary supplement which specifies an amount of said at least one of said one or more active components determined in step ii), as part of said quantitative compositional fingerprint comparison.

15 18. The method of claim 1, wherein said fractions are prepared using a first method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas

20 chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

19. The method of claim 17, wherein said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

20. The method of claim 17, wherein:  
said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and  
said second method is identical to said first method.

21. The method of claim 17, wherein:  
said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and

said second method is different from said first method.

22. The method of claim 1, further comprising:

i) determining from among said plurality of fractions the amount in said extract of at

5 least one inactive component, said at least one inactive component having essentially no activity in said active component bioassay;

ii) comparing the amount of said at least one inactive component determined in step i) to an inactive component standard for a functional food grade supplement which specifies an amount of said at least one inactive component determined in step i), thereby providing a  
10 inactive component fingerprint comparison; and

(iii) determining whether said extract is a standardized functional food product or a standardized dietary supplement based on said bioactivity comparison, said quantitative compositional fingerprint comparison, and said inactive component fingerprint comparison.

15 23. A method for standardizing a botanical extract to create a standardized functional food product or a standardized dietary supplement, comprising the steps of:

a) removing at least one first aliquot from said extract;

b) determining from said at least one first aliquot a bioactivity value for said extract, using an extract bioassay to measure said bioactivity value;

20 c) comparing said bioactivity value for said extract to a bioactivity standard that specifies one of a minimal total bioactivity, a maximal total bioactivity, and a range of total bioactivities required for a standardized functional food product or a standardized dietary supplement as measured using said extract bioassay to provide a bioactivity comparison;

d) removing at least one second aliquot from said extract;

e) fractionating said at least one second aliquot into a plurality of fractions, said plurality of fractions containing one or more active components;

f) determining from among said plurality of fractions the bioactivity in said extract of at least one of said one or more active components having activity in an active component bioassay;

g) comparing the bioactivity of said at least one of said one or more active components determined in step f) to an active component bioactivity standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of said at least one of said one or more active components to provide a component bioactivity fingerprint comparison; and

(h) determining whether said extract is a standardized functional food product or a standardized dietary supplement based on said bioactivity comparison and said component bioactivity fingerprint comparison.

24. The method of claim 23, wherein said botanical extract is an extract of soy.

25. The method of claim 23, wherein said botanical extract is an extract of a member selected from the group consisting of wheat bran, gelatin, and cranberries.

26. The method of claim 24, wherein said extract of soy is one of soy meal, soy flakes, defatted soy flakes, soy sauce, soybean oil, tofu, soy milk, soy flour, soy nuts, tempeh, miso, and natto.

27. The method of claim 23, wherein said extract bioassay is one of adrenergic  $\alpha 1$  receptor binding, NS; adrenergic  $\alpha 2$  receptor binding, NS; adrenergic  $\beta$  receptor binding, NS; human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptor binding; EGF receptor binding; EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2 tyrosine kinase activity; HMG CoA reductase activity; Ca<sup>++</sup>-dependent K<sup>+</sup> channel binding; IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity; constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>lyn</sup> tyrosine kinase; p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane A<sub>2</sub>-induced platelet aggregation; thyrotropin releasing hormone receptor binding; TNF receptor binding; and xanthine oxidase activity.

28. The method of claim 23, wherein said active component bioassay is one of adrenergic  $\alpha 1$  receptor binding, NS; adrenergic  $\alpha 2$  receptor binding, NS; adrenergic  $\beta$  receptor binding, NS; human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptor binding; EGF receptor binding; EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2 tyrosine kinase activity; HMG CoA reductase activity; Ca<sup>++</sup>-dependent K<sup>+</sup> channel binding; IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity; constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>lyn</sup> tyrosine kinase; p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane A<sub>2</sub>-

induced platelet aggregation; thyrotropin releasing hormone receptor binding; TNF receptor binding; and xanthine oxidase activity.

5        29.     The method of claim 23, wherein said extract bioassay is the same as said active component bioassay.

30.     The method of claim 23, wherein said extract bioassay is different from said active component bioassay.

10       31.     The method of claim 23, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cancer.

32.     The method of claim 23, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to osteoporosis.

15       33.     The method of claim 23, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to premenstrual tension syndrome.

34.     The method of claim 23, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to postmenopausal symptoms.

20       35.     The method of claim 23, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cardiovascular diseases.



36. The method of claim 23, wherein at least one of said plurality of different fractions contains a single essentially purified chemical compound.

37. The method of claim 23, wherein said steps a) through h) are repeated at least once.

38. The method of claim 23, further comprising:

i) fractionating at least one of said plurality of fractions into a plurality of subfractions, said plurality of subfractions containing one or more active components;

ii) determining from among said plurality of subfractions the amount in said extract of at least one of said one or more active components, said at least one of said one or more active components having activity in said active component bioassay; and

iii) comparing the amount of said at least one of said one or more active components determined in step ii) to a standard for a standardized functional food product or a standardized dietary supplement which specifies an amount of said at least one of said one or more active components determined in step ii), as part of said quantitative compositional fingerprint comparison.

39. The method of claim 23, wherein said fractions are prepared using a first method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, and chromatographic separation techniques, such as flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer

chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

40. The method of claim 38, wherein said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

41. The method of claim 38, wherein:

said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and said second method is identical to said first method.

42. The method of claim 38, wherein:

said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid

chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and said second method is different from said first method.

5

43. A method for standardizing a botanical extract to create a standardized functional food product or a standardized dietary supplement, comprising the steps of:

- a) removing at least one first aliquot from said extract;
- b) determining from said at least one first aliquot a bioactivity value for said extract,

10 using an extract bioassay to measure said bioactivity value;

c) comparing said bioactivity value for said extract to a bioactivity standard that specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities required for a standardized functional food product or a standardized dietary supplement as measured using said bioassay to provide a bioactivity comparison;

15 d) removing at least one second aliquot from said extract;

e) fractionating said at least one second aliquot into a plurality of fractions, said plurality of fractions containing one or more active components;

f) determining from among said plurality of fractions the bioactivity and the amount in said extract of at least one of said one or more active components having activity in said an  
20 active component bioassay;

g) comparing the amount of said at least one of said one or more active components determined in step f) to an active component standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal amount, a

maximal amount, and a range of amounts of said at least one of said one or more active components to provide a quantitative compositional fingerprint comparison;

h) comparing the bioactivity of said at least one of said one or more active components determined in step f) to an active component bioactivity standard for a standardized functional food product or a standardized dietary supplement which specifies one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of said at least one of said one or more active components to provide a component bioactivity fingerprint comparison; and

(i) determining whether said extract is a standardized functional food product or a standardized dietary supplement based on said bioactivity comparison, said component bioactivity fingerprint comparison, and said quantitative compositional fingerprint comparison.

44. The method of claim 43, wherein said botanical extract is an extract of soy.

45. The method of claim 43, wherein said botanical extract is an extract of a member selected from the group consisting of wheat bran, gelatin, and cranberries.

46. The method of claim 44, wherein said extract of soy is one of soy meal, soy flakes, defatted soy flakes, soy sauce, soybean oil, tofu, soy milk, soy flour, soy nuts, tempeh, miso, and natto.

47. The method of claim 43, wherein said bioactivity specified by said bioactivity standard is one of adrenergic  $\alpha 1$  receptor binding, NS; adrenergic  $\alpha 2$  receptor binding, NS;

adrenergic  $\beta$  receptor binding, NS; human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptor binding; EGF receptor binding; EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2 tyrosine kinase activity; HMG CoA reductase activity; Ca<sup>++</sup>-dependent K<sup>+</sup> channel binding; IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity; constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>fm</sup> tyrosine kinase; p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane A<sub>2</sub>-induced platelet aggregation; thyrotropin releasing hormone receptor binding; TNF receptor binding; and xanthine oxidase activity.

48. The method of claim 43, wherein said active component bioassay is one of adrenergic  $\alpha$ 1 receptor binding, NS; adrenergic  $\alpha$ 2 receptor binding, NS; adrenergic  $\beta$  receptor binding, NS; human dopamine D<sub>2L</sub> (D<sub>2A</sub>) receptor binding; EGF receptor binding; EGF receptor tyrosine kinase activity; ERK serine/threonine kinase activity; estrogen receptor  $\alpha$  binding; estrogen receptor  $\beta$  binding; glucocorticoid receptor binding; HER2 tyrosine kinase activity; HMG CoA reductase activity; Ca<sup>++</sup>-dependent K<sup>+</sup> channel binding; IL-6 receptor binding; IL-8 receptor binding; lipid peroxidation; MAP kinase activity; constitutive nitric oxide synthase activity; oxytocin receptor binding; p59<sup>fm</sup> tyrosine kinase; p56<sup>lck</sup> tyrosine kinase; PAF receptor binding; progesterone receptor binding; protein kinase A activity, NS; superoxide dismutase activity; testosterone receptor binding; thromboxane A<sub>2</sub>-induced platelet aggregation; thyrotropin releasing hormone receptor binding; TNF receptor binding; and xanthine oxidase activity.

49. The method of claim 43, wherein said active component is one or more of daidzein; genistein; glycitein; daidzin; genistin; glycitin; 6-O-acetyl daidzin; 6-O-acetyl genistin; 6-O-acetyl glycitin; 6-O-malonyl daidzin; 6-O-malonyl genistin; 6-O-malonyl glycitin; L- $\alpha$ -lysophosphatidylcholine; L- $\alpha$ -phosphatidylcholine; L- $\alpha$ -phosphatidylethanolamine; L- $\alpha$ -phosphatidylinositol; L- $\alpha$ -phosphatidylserine; campesterol;  $\beta$ -sitostanol;  $\beta$ -sitosterol; stigmasterol; coumestrol;  $\alpha$ -tocopherol; and Bowman-Birk trypsin inhibitor.

50. The method of claim 43, wherein said extract bioassay is the same as said active component bioassay.

51. The method of claim 43, wherein said extract bioassay is different from said active component bioassay.

52. The method of claim 43, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cancer.

53. The method of claim 43, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to osteoporosis.

54. The method of claim 43, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to premenstrual tension syndrome.

55. The method of claim 43, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to postmenopausal symptoms.

56. The method of claim 43, wherein at least one of said extract bioassay and said active component bioassay measure bioactivities linked to cardiovascular diseases.

57. The method of claim 43, wherein at least one of said plurality of different fractions contains a single essentially purified chemical compound.

58. The method of claim 43, wherein said steps a) through h) are repeated at least once.

59. The method of claim 43, further comprising:

i) fractionating at least one of said plurality of fractions into a plurality of subfractions, said plurality of subfractions containing one or more active components;

ii) determining from among said plurality of subfractions the amount in said extract of at least one of said one or more active components, said at least one of said one or more active components having activity in said active component bioassay; and

iii) comparing the amount of said at least one of said one or more active components determined in step ii) to a standard for a standardized functional food product or a standardized dietary supplement which specifies an amount of said at least one of said one or more active components determined in step ii), as part of said quantitative compositional fingerprint comparison.

60. The method of claim 43, wherein said fractions are prepared using a first method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

61. The method of claim 59, wherein said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography.

62. The method of claim 59, wherein:

said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and

said second method is identical to said first method.



63. The method of claim 59, wherein:

said subfractions are prepared using a second method selected from the group consisting of liquid/liquid extractions, pH-dependent separations, isoelectric focusing, preparative electrophoresis, flash chromatography, preparative high performance liquid chromatography (HPLC), preparative gas chromatography, preparative thin-layer chromatography (TLC), affinity chromatography, size exclusion chromatography, countercurrent chromatography, and centripetal or centrifugal chromatography; and

said second method is different from said first method.

64. The method of claim 43, further comprising:

i) determining from among said plurality of fractions the amount in said extract of at least one inactive component, said at least one inactive component having essentially no activity in said active component bioassay;

ii) comparing the amount of said at least one inactive component determined in step i) to an inactive component standard for a standardized functional food product or a standardized dietary supplement which specifies an amount of said at least one inactive component determined in step i), thereby providing a inactive component fingerprint comparison; and

(iii) determining whether said extract is a standardized functional food product or a standardized dietary supplement based on said bioactivity comparison, said quantitative compositional fingerprint comparison, and said inactive component fingerprint comparison.

65. A method for establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, comprising the steps of:

a) establishing a botanical extract;

b) fractionating an aliquot of said extract into a plurality of fractions, said plurality of fractions containing substantially all the mass of said aliquot;

c) ascertaining an amount of at least one active component in at least one of said plurality of fractions;

d) specifying an active component standard for said fingerprint standard, said active component fingerprint standard including one of a minimal amount, a maximal amount, and a range of amounts of said at least one active component in said at least one of said plurality of fractions;

e) ascertaining a bioactivity value of said extract, using an extract bioassay;

f) specifying a bioactivity standard for said fingerprint standard, said bioactivity standard including one of a minimal bioactivity value, a maximal bioactivity value, and a range of bioactivity values in said extract for said extract bioassay; and

g) establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, said fingerprint standard comprising said active component standard and said bioactivity standard.

66. A method for establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, comprising the steps of:

a) establishing a botanical extract;

b) fractionating an aliquot of said extract into a plurality of fractions, said plurality of fractions containing substantially all the mass of said aliquot;

c) ascertaining a bioactivity of at least one active component in at least one of said plurality of fractions;

d) specifying an active component bioactivity standard for said fingerprint standard, said active component bioactivity standard including one of a minimal bioactivity, a maximal bioactivity, and a range of bioactivities of said at least one active component in said at least one of said plurality of fractions;

e) ascertaining a bioactivity value of said extract, using an extract bioassay;

f) specifying a bioactivity standard for said fingerprint standard, said bioactivity standard including one of a minimal bioactivity value, a maximal bioactivity value, and a range of bioactivity values in said extract for said extract bioassay; and

g) establishing a fingerprint standard for a standardized functional food product or a standardized dietary supplement, said fingerprint standard comprising said active component bioactivity standard and said bioactivity standard.

67. A method for preparing a standardized functional food product or a standardized dietary supplement, comprising the steps of:

a) preparing a plurality of extracts from at least one botanical;

b) screening each of said plurality of extracts against a plurality of bioassays, each of said plurality of bioassays being associated with a target physiological condition;

c) identifying one or more of said plurality of extracts having a desired level of bioactivity in at least one of said plurality of bioassays;

d) determining the level of bioactivity in each of said plurality of extracts identified in step c) for each of said plurality of bioassays; and

e). mixing two or more of said plurality of extracts to form a standardized functional food product or a standardized dietary supplement, said standardized functional food product or standardized dietary supplement having a desired level of bioactivity for each of said plurality of bioassays associated with said target physiological condition.

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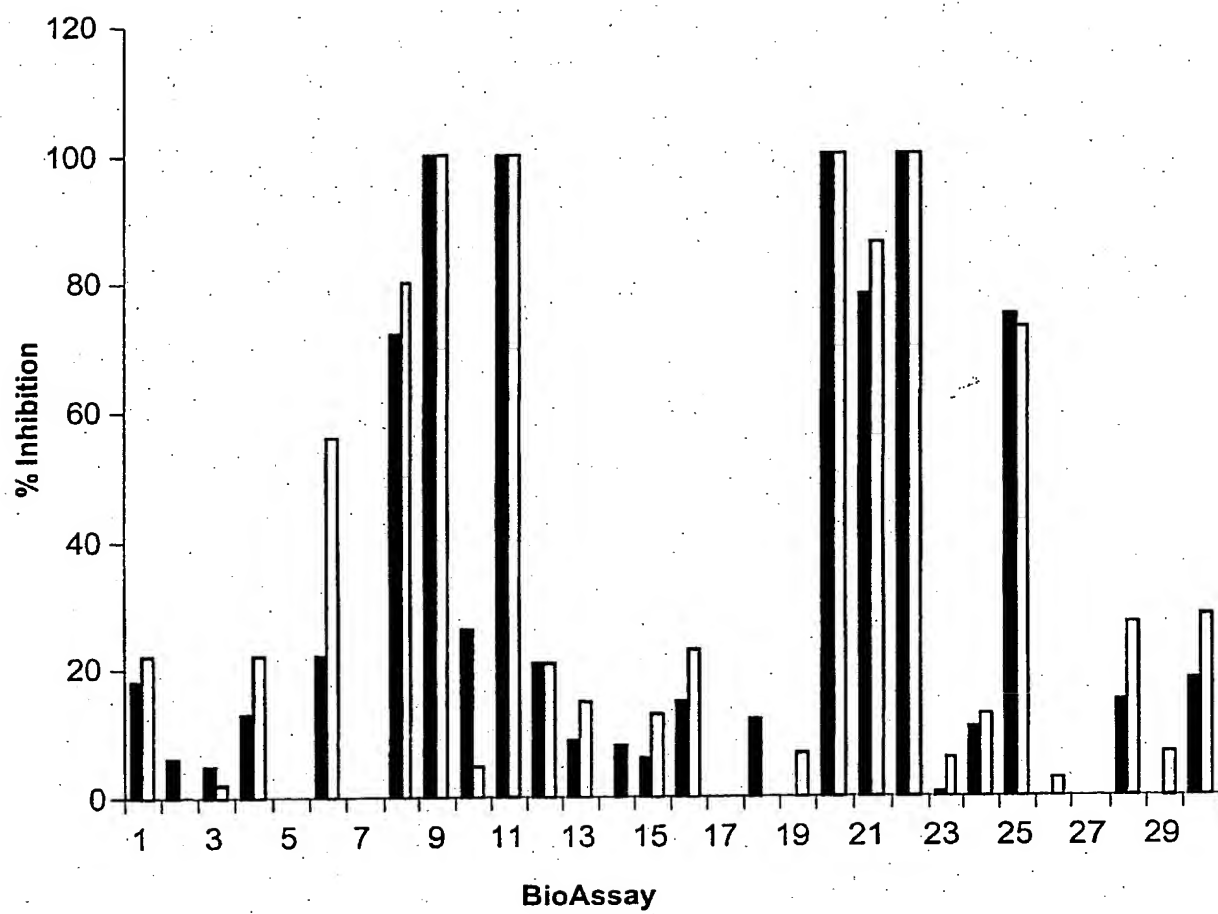


Figure 1A

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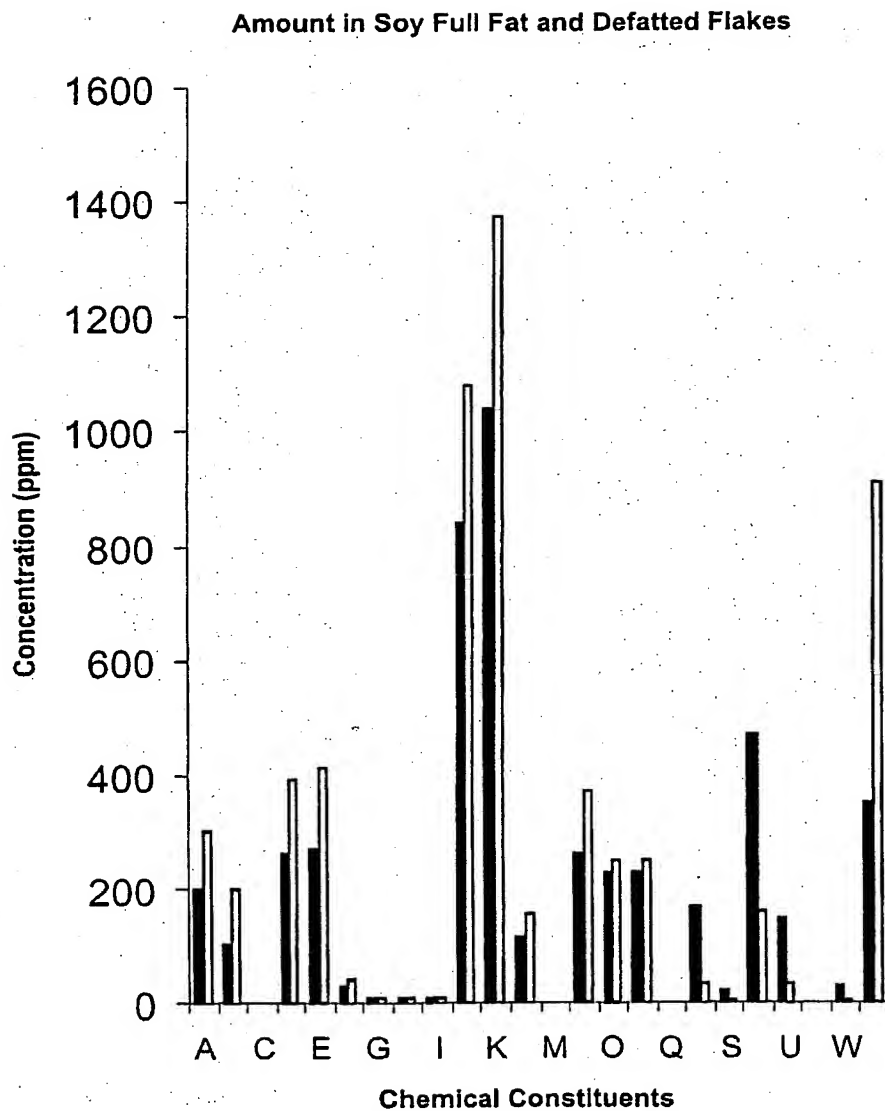


Figure 1B

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# ER beta-Aqueous

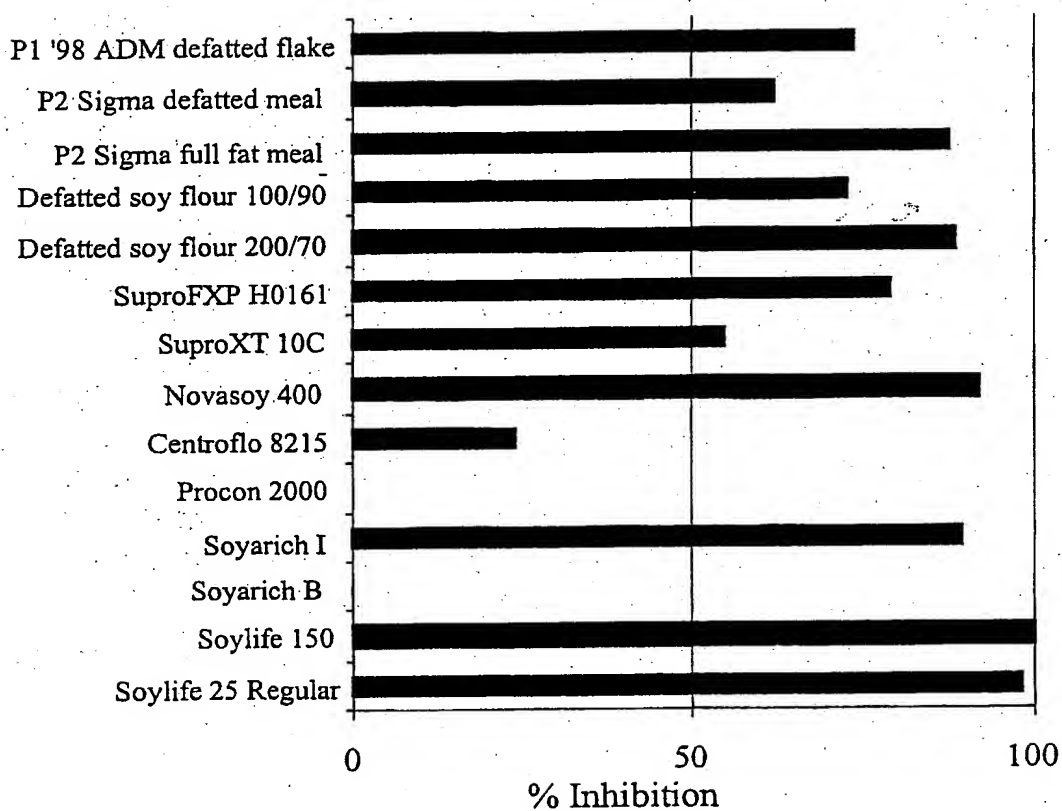


Figure 2A

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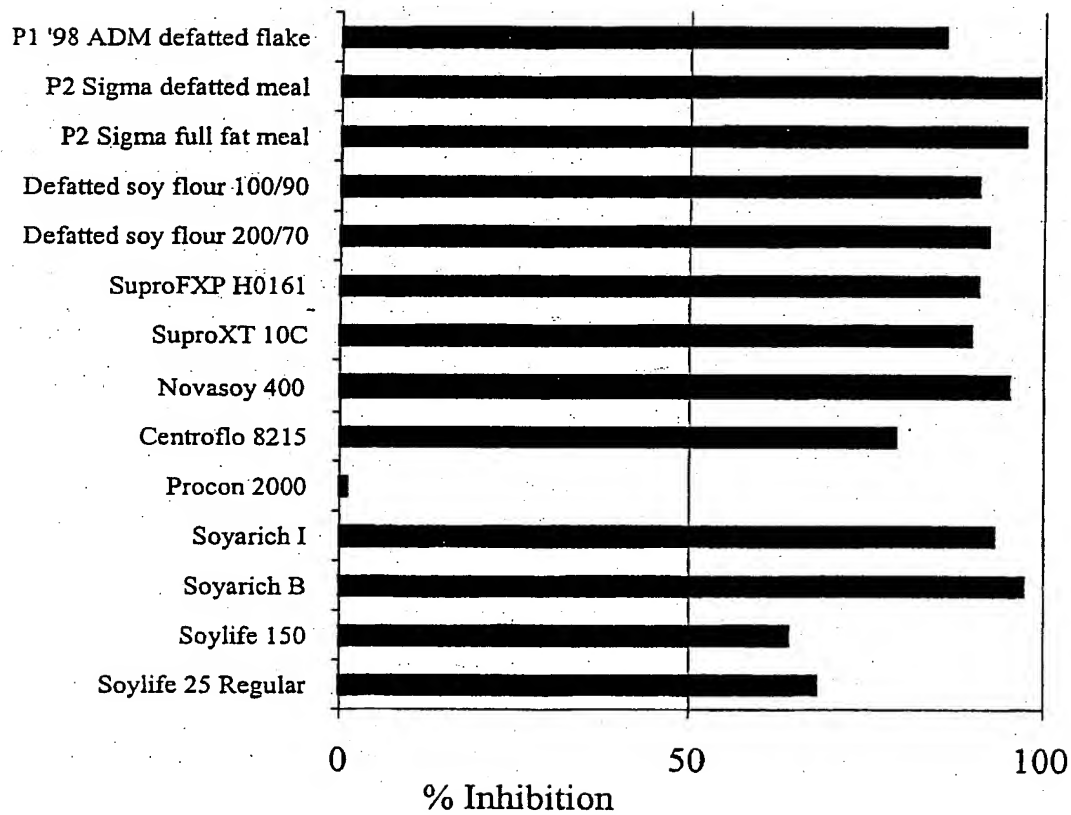
**p59<sup>fyn</sup> TK-Aqueous**

Figure 2B



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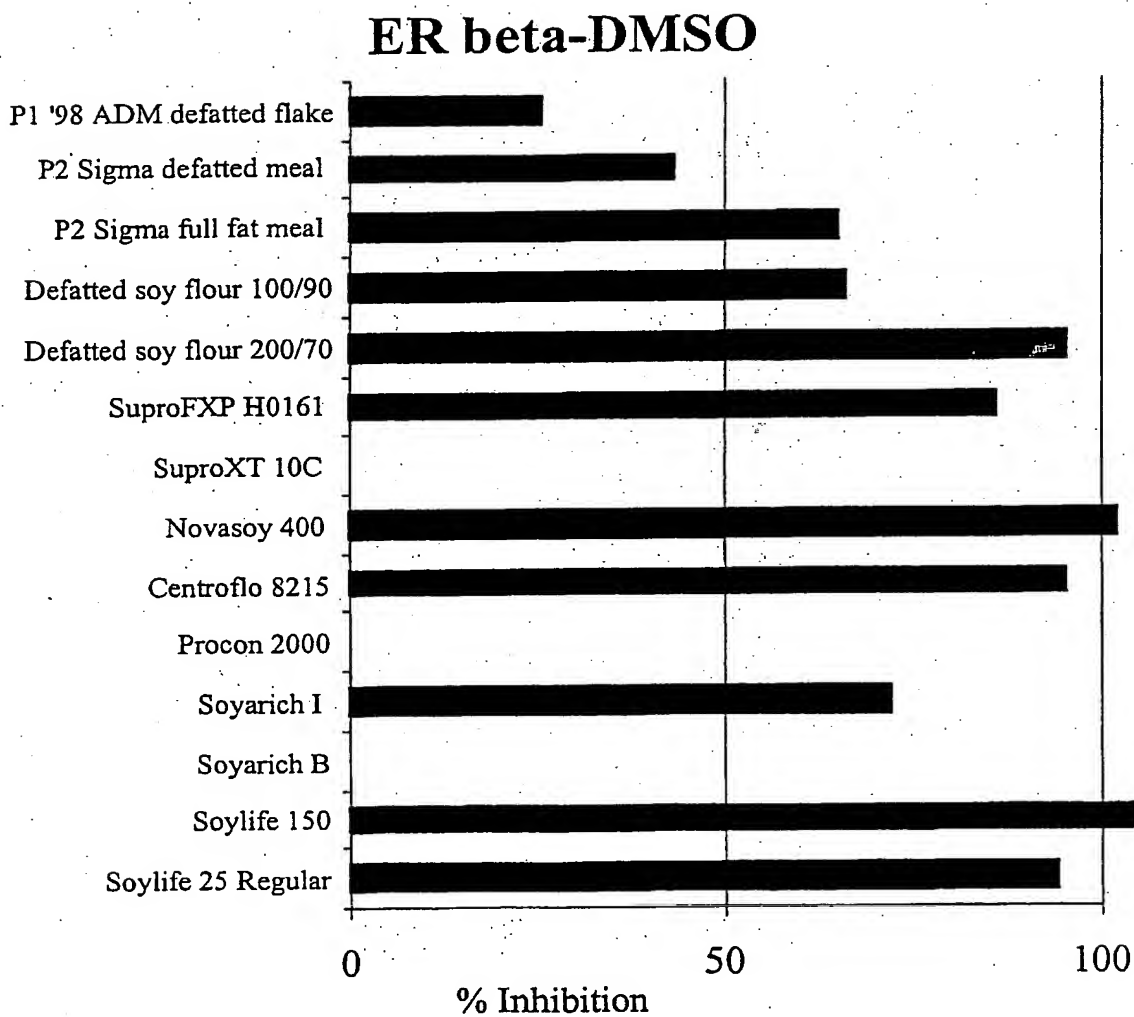
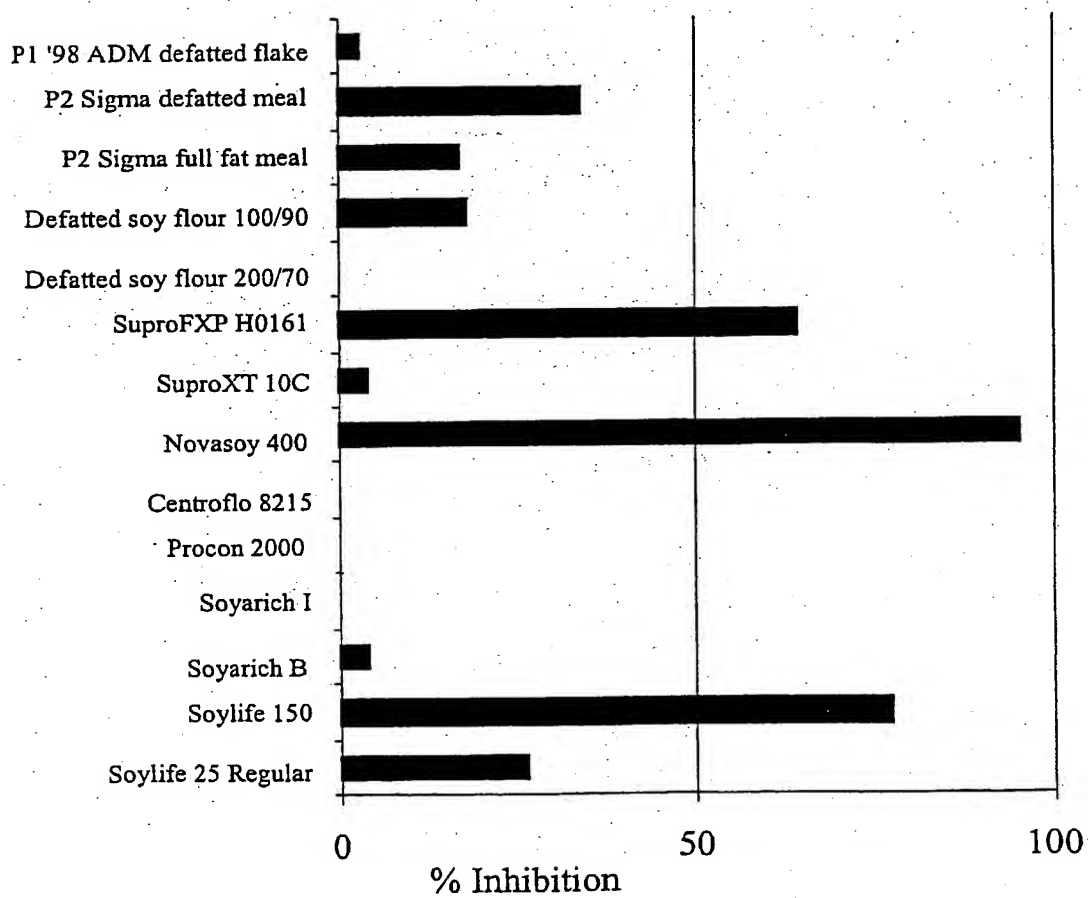
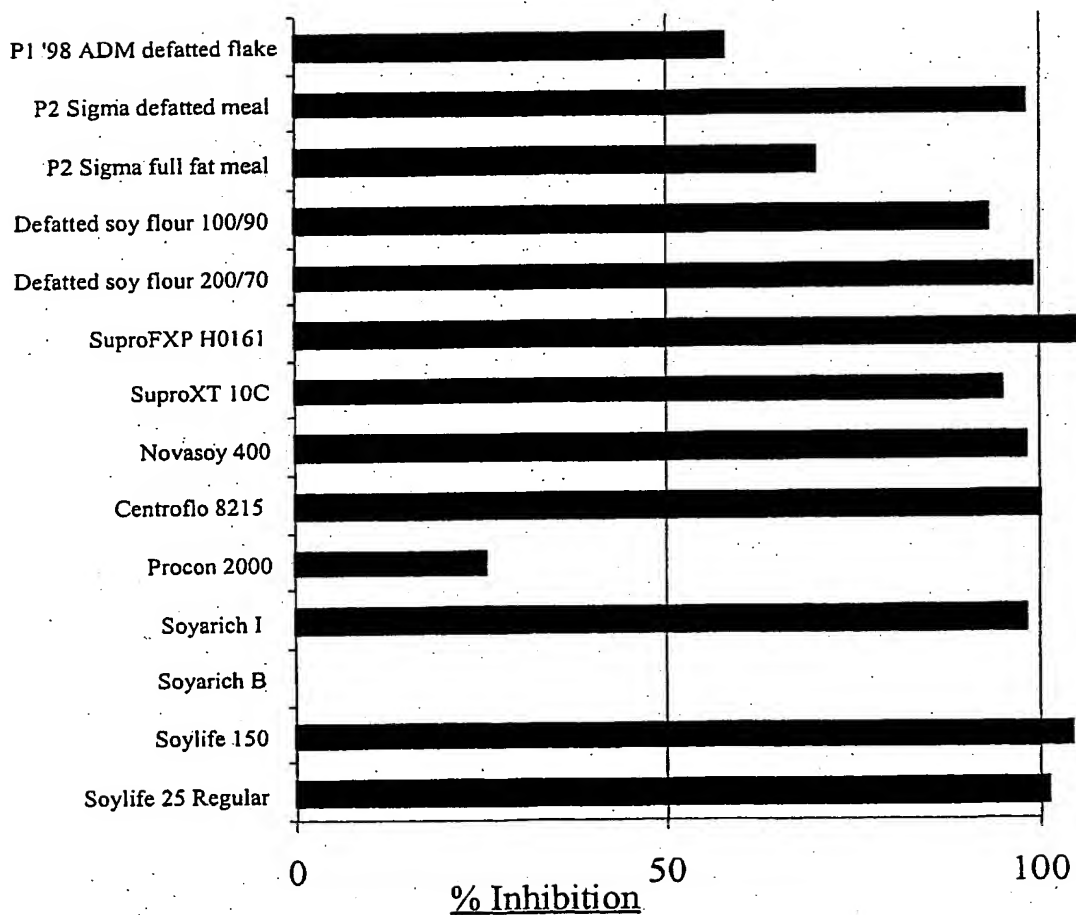


Figure 2C

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**P59<sup>fyn</sup> TK-DMSO****Figure 2D**

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**ER beta-Ethanol****Figure 2E**

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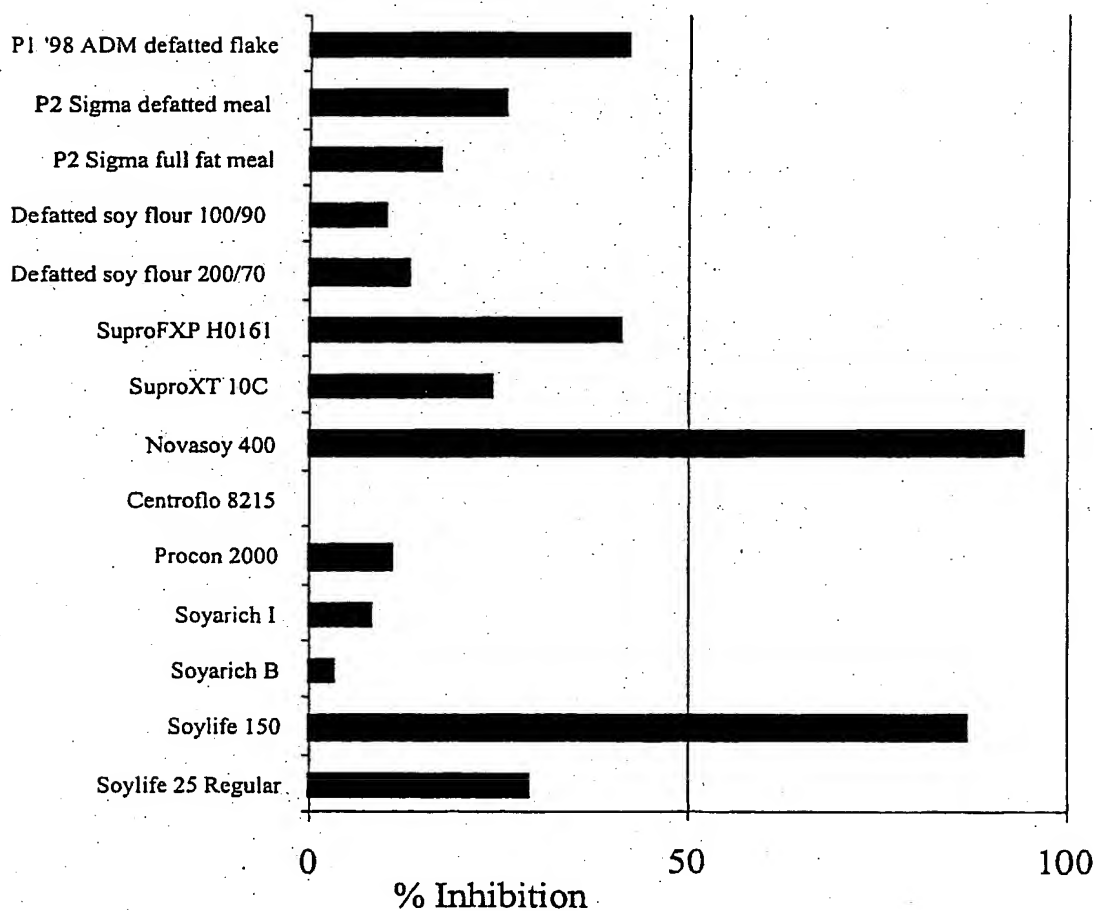
**p59<sup>syn</sup> TK-Ethanol**

Figure 2F

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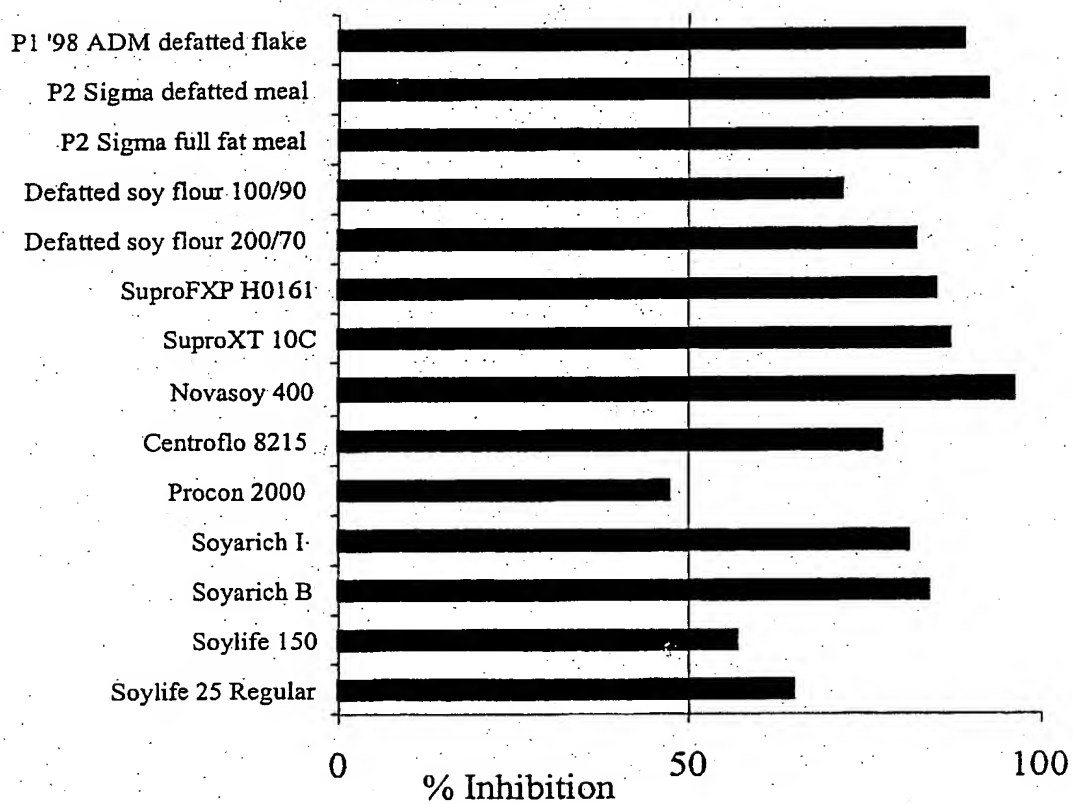
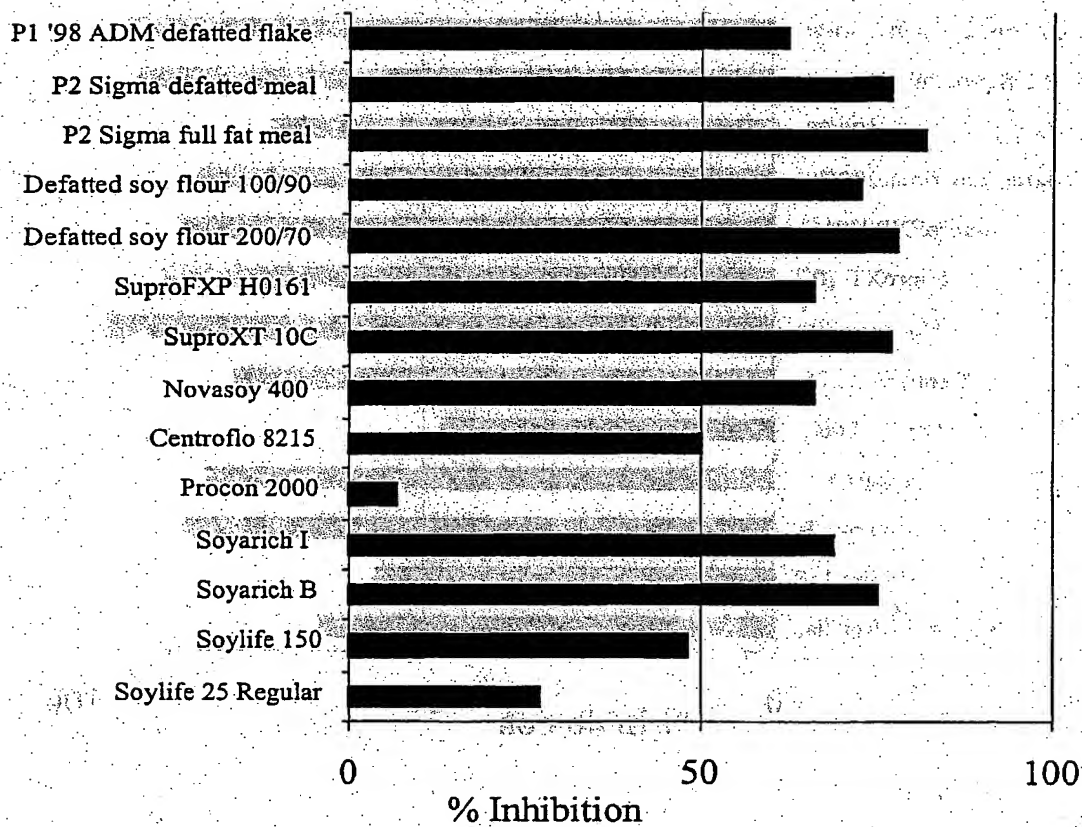
**p56<sup>lck</sup> TK-Aqueous**

Figure 3A

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**EGF-R-Aqueous****Figure 3B**

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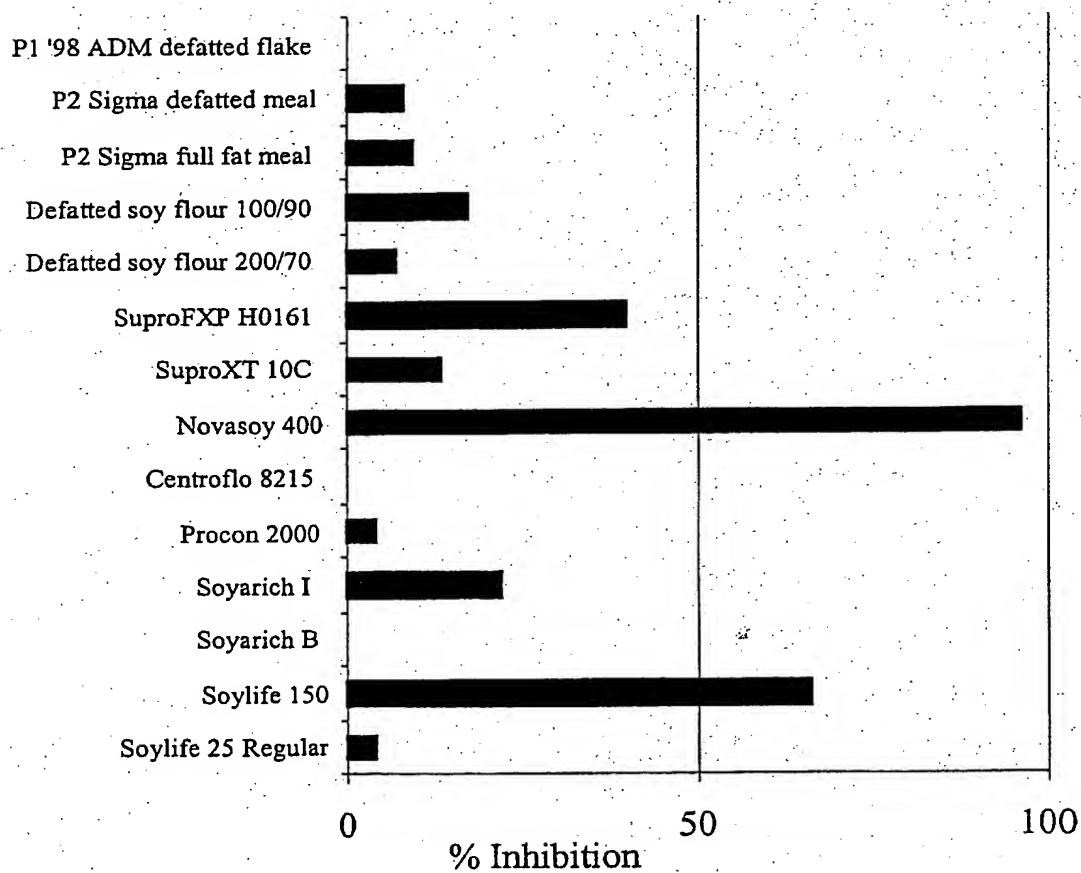
p56<sup>lck</sup> TK-DMSO

Figure 3C

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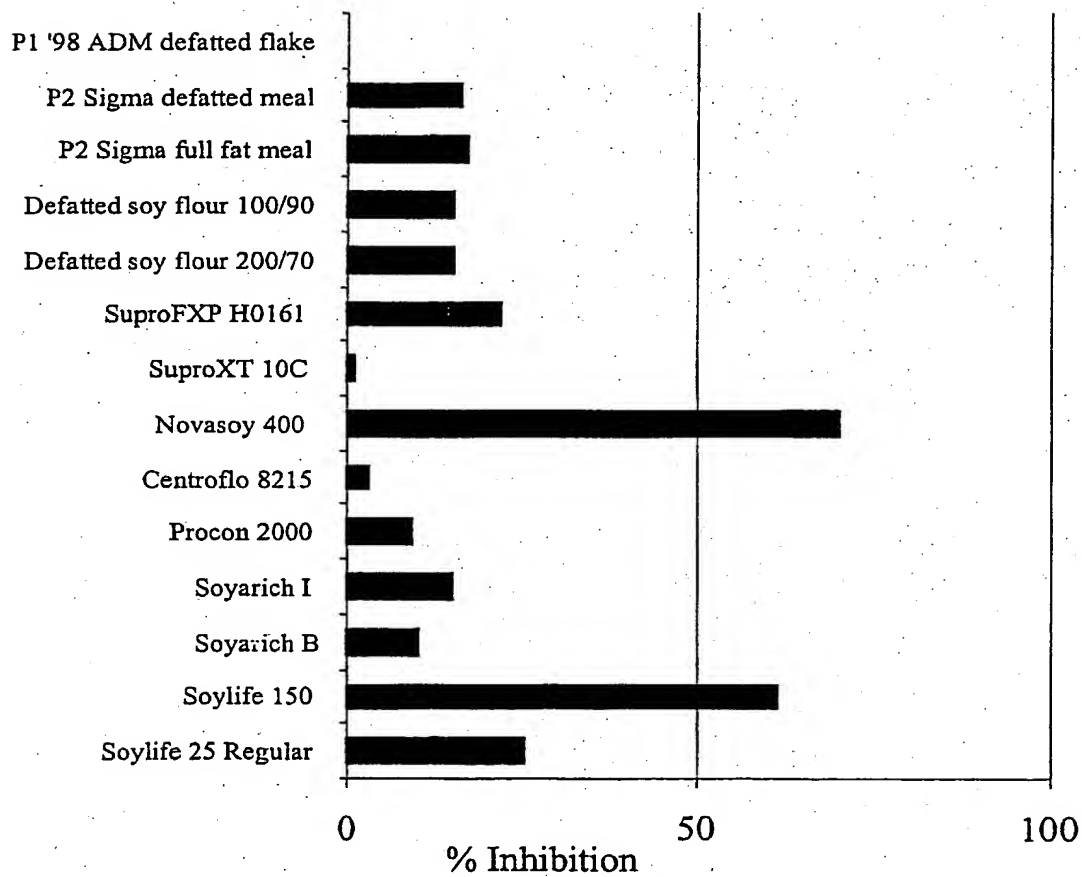
**EGF-R-DMSO**

Figure 3D



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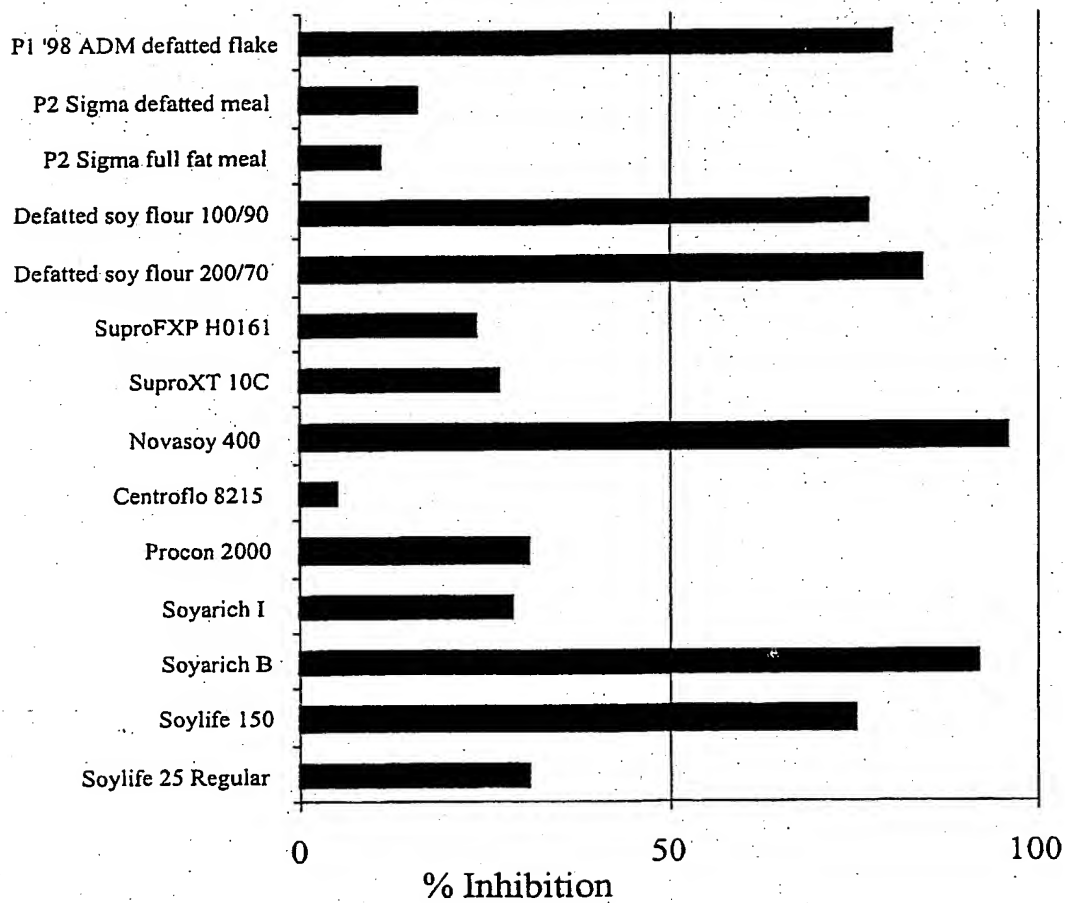
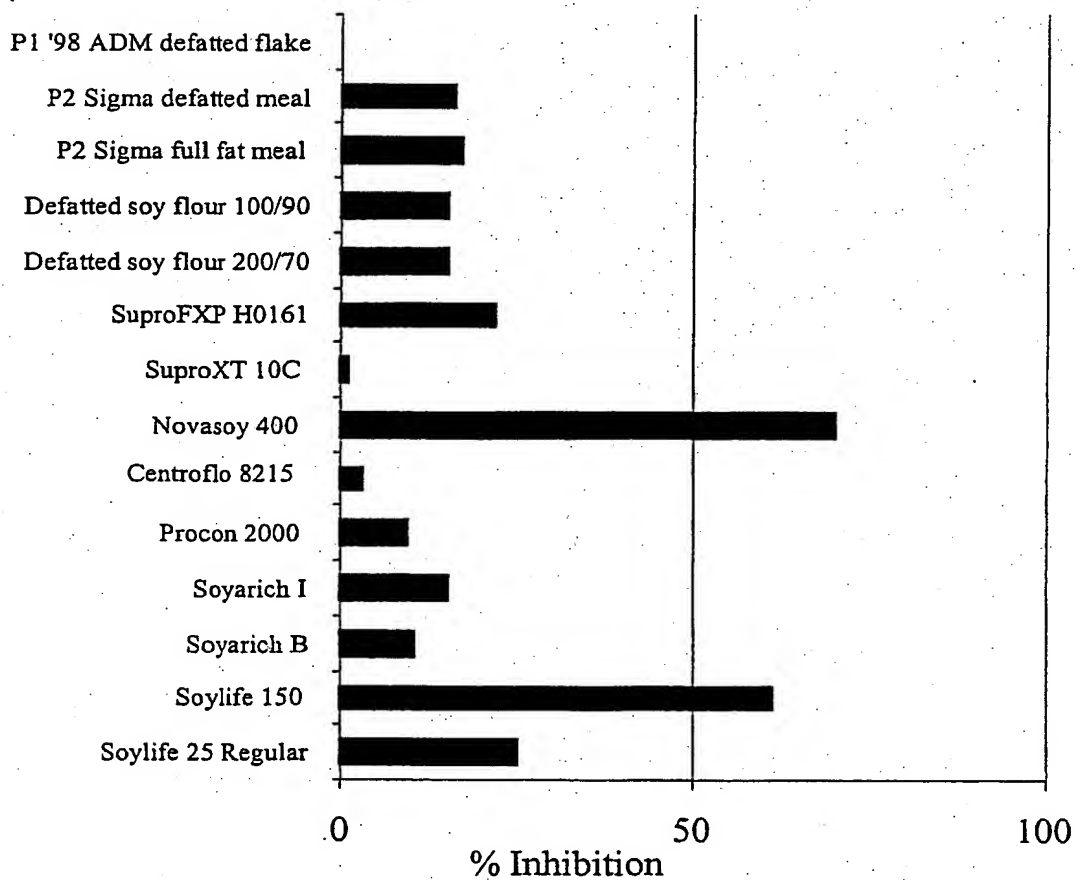
**p56<sup>lck</sup> TK-Ethanol**

Figure 3E

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**EGF-R-DMSO****Figure 3F**

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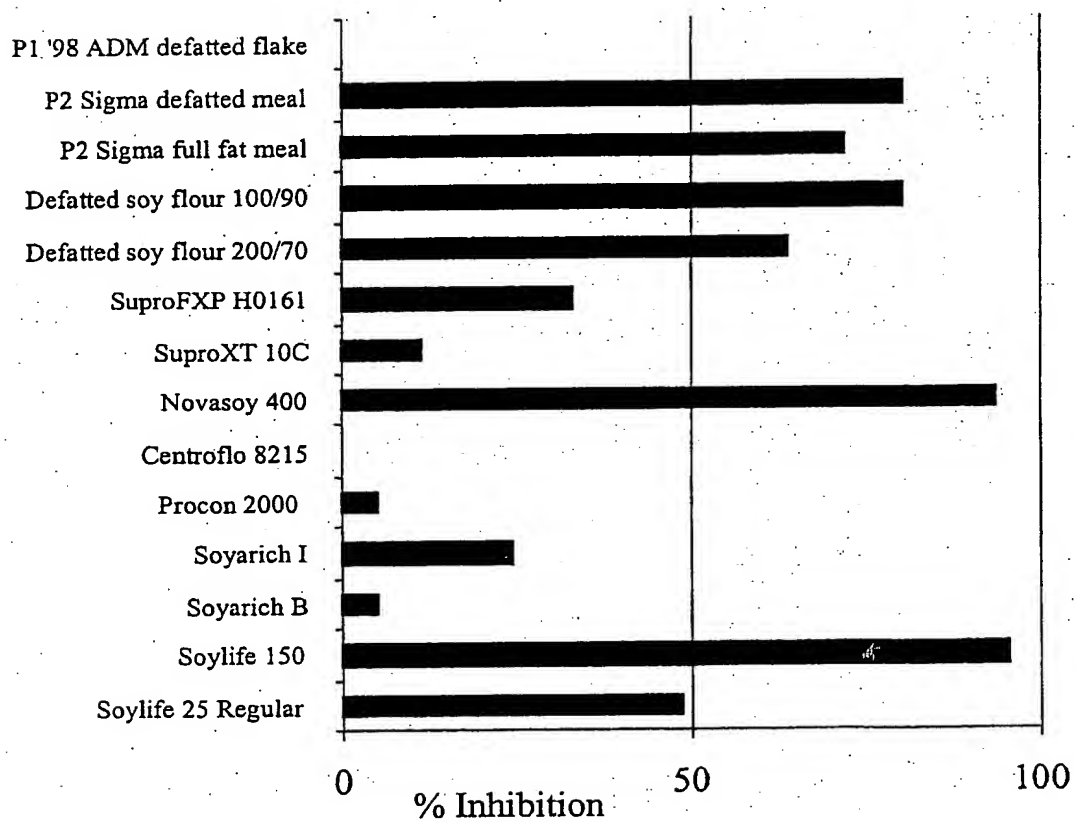
**ER alpha-Aqueous**

Figure 4A

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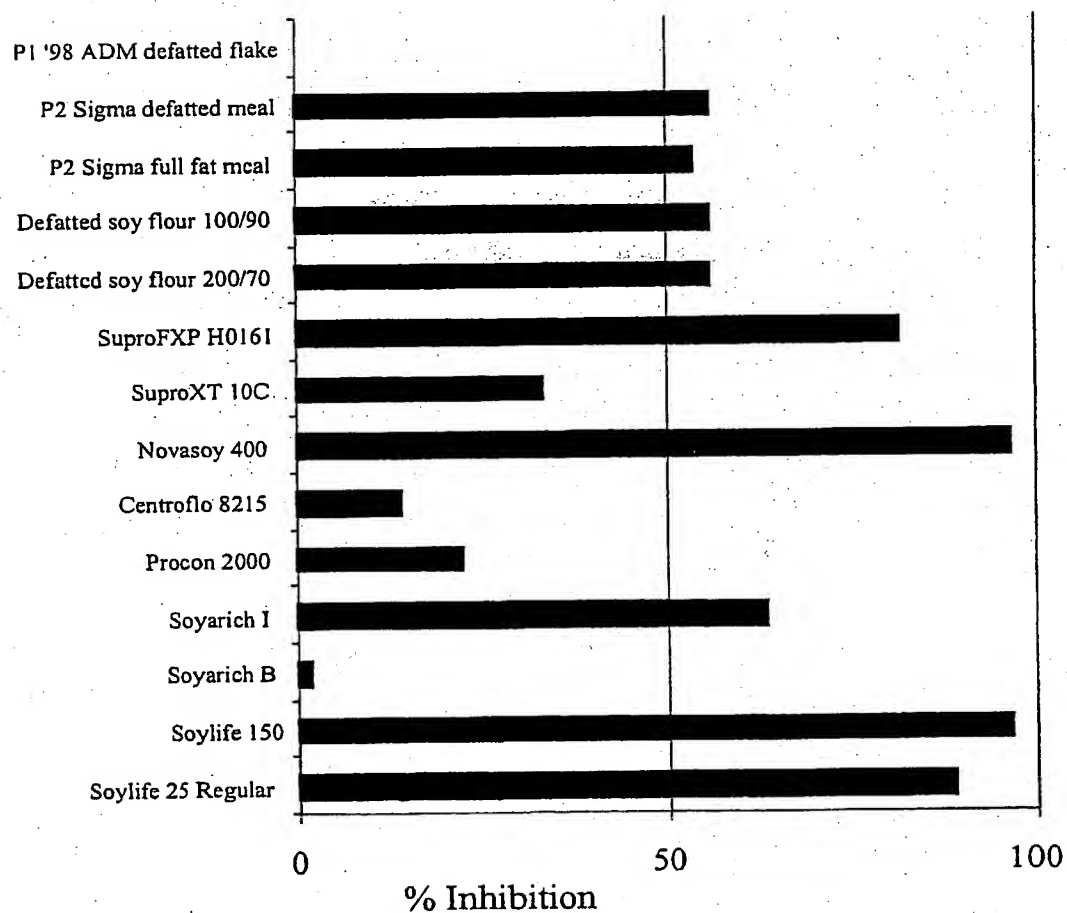
**ER alpha-Ethanol**

Figure 4B

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## HMG CoA Reductase-Aqueous

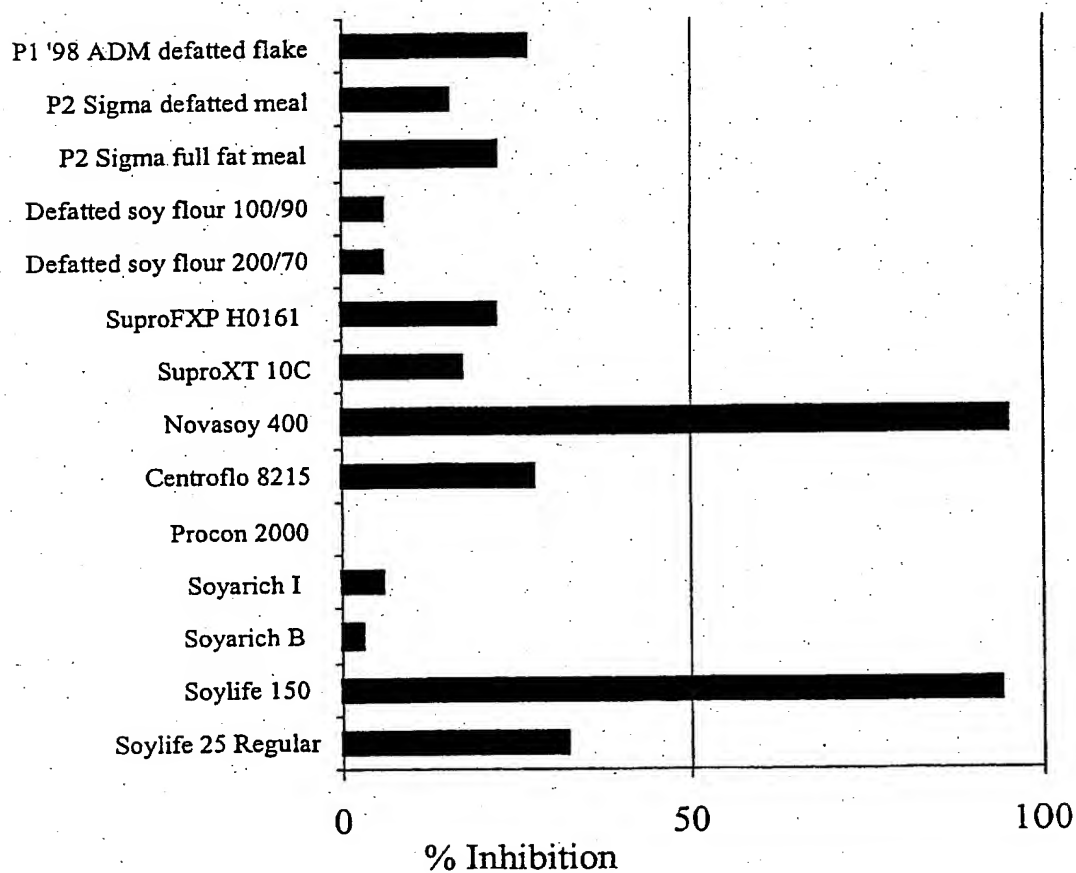
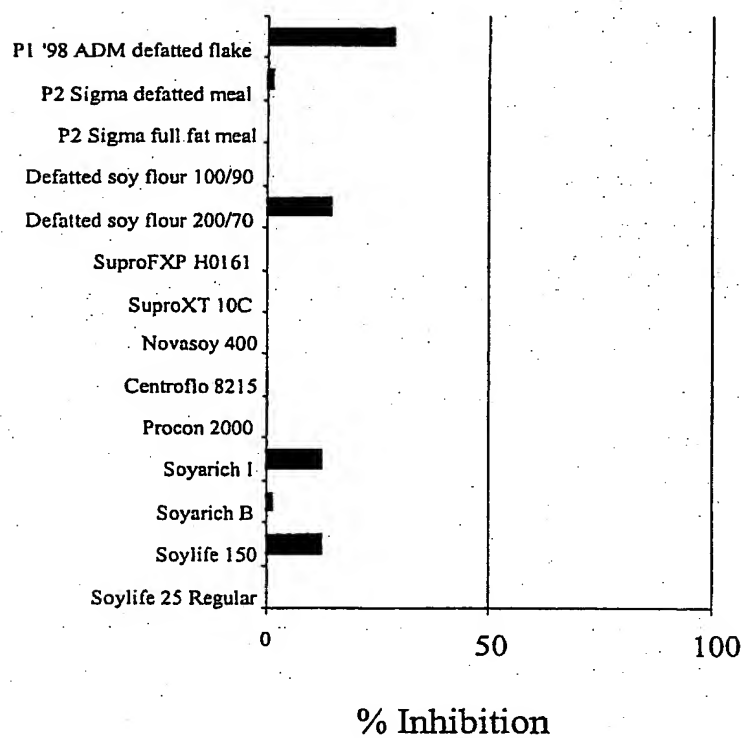


Figure 5A

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**Lipid Peroxidation-Aqueous****Figure 5B**

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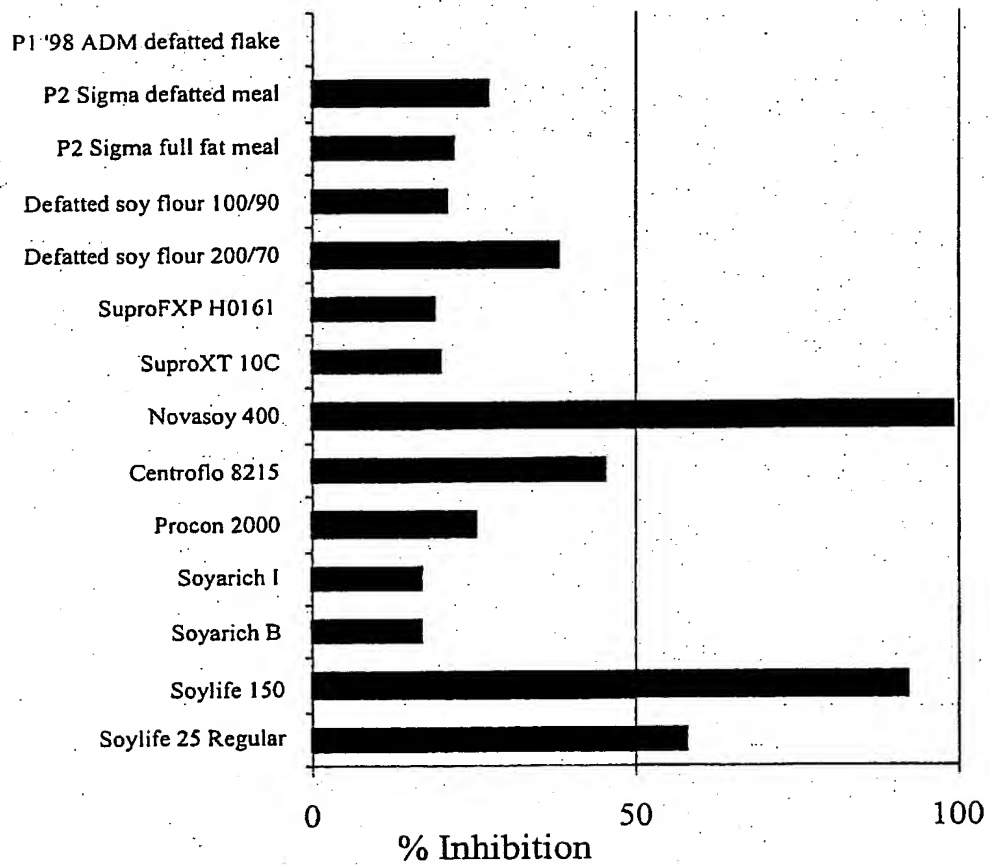
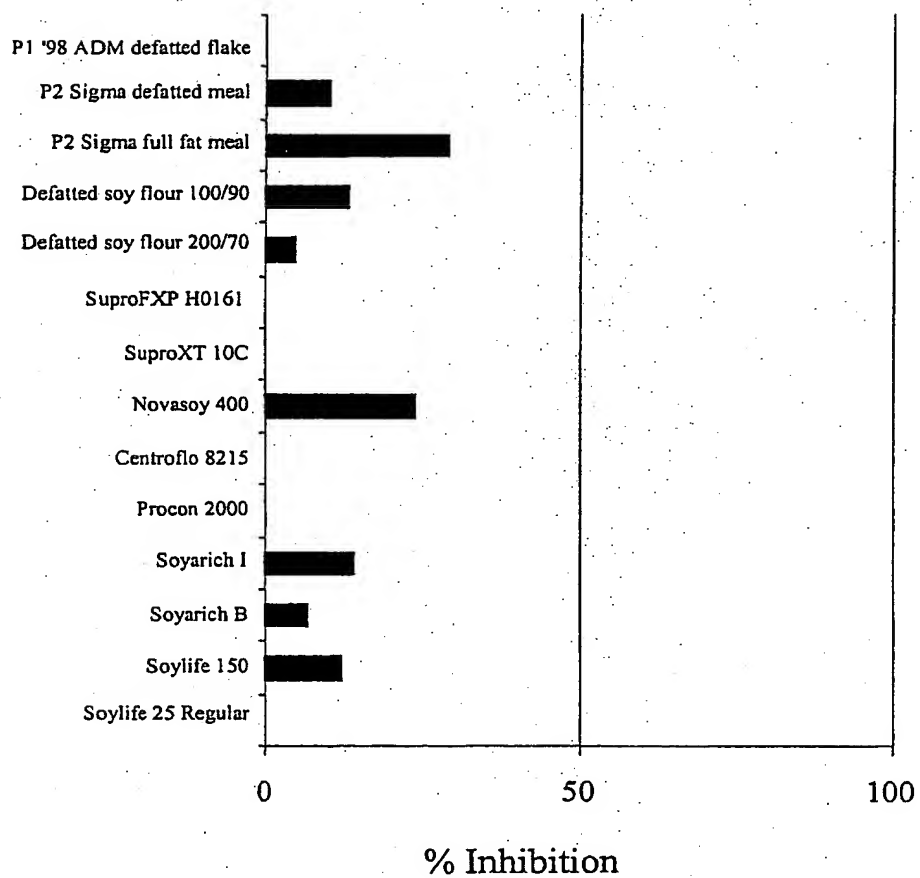
**HMG CoA Reductase-DMSO**

Figure 5C

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**Lipid Peroxidation-DMSO****Figure 5D**



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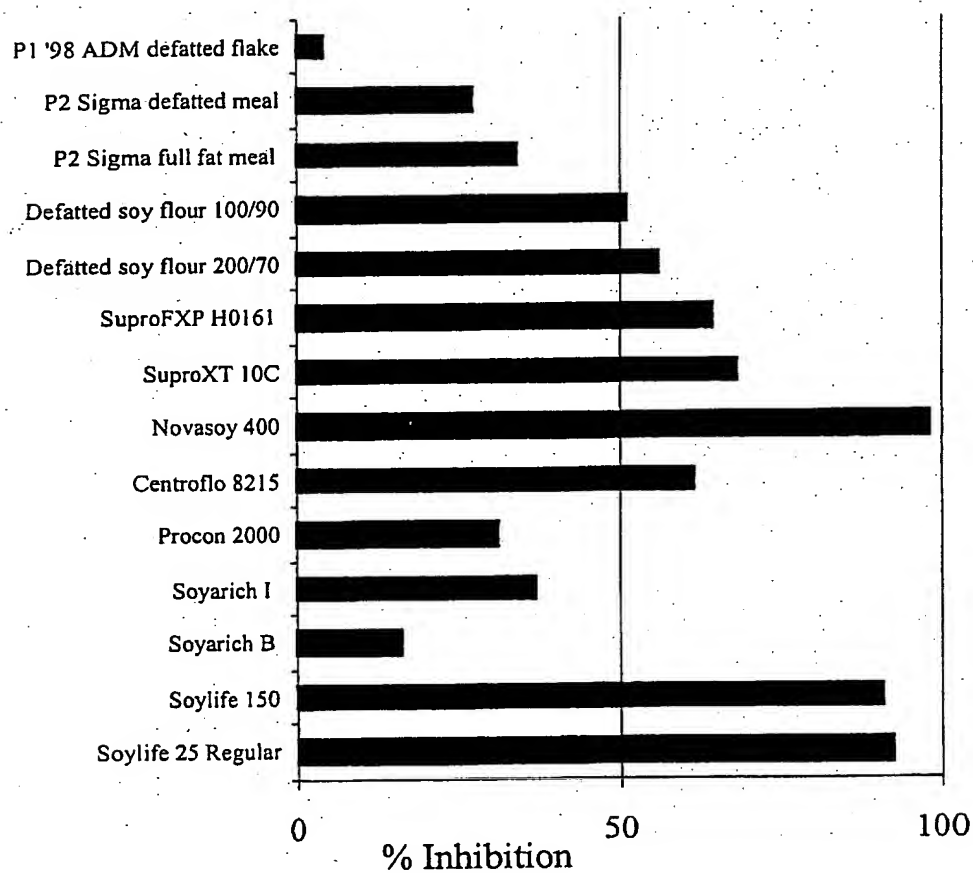
**HMG CoA Reductase-Ethanol**

Figure 5E

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### Lipid Peroxidation-Ethanol

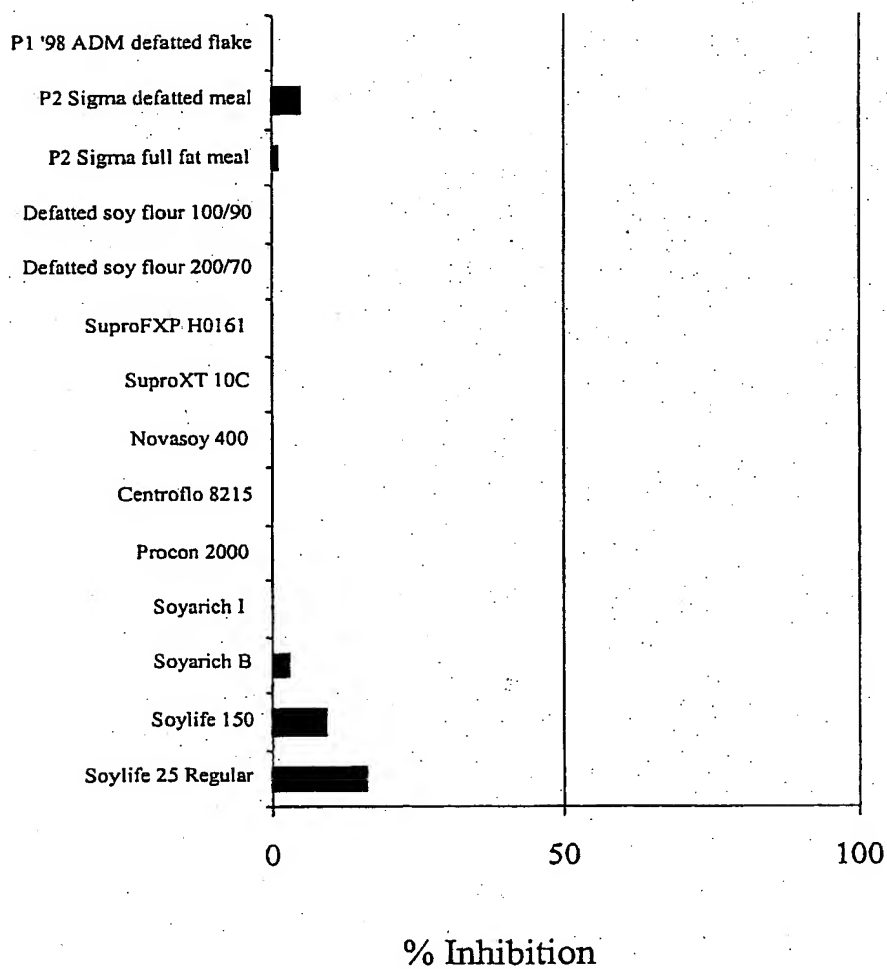


Figure 5F

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# PAF Binding-Aqueous

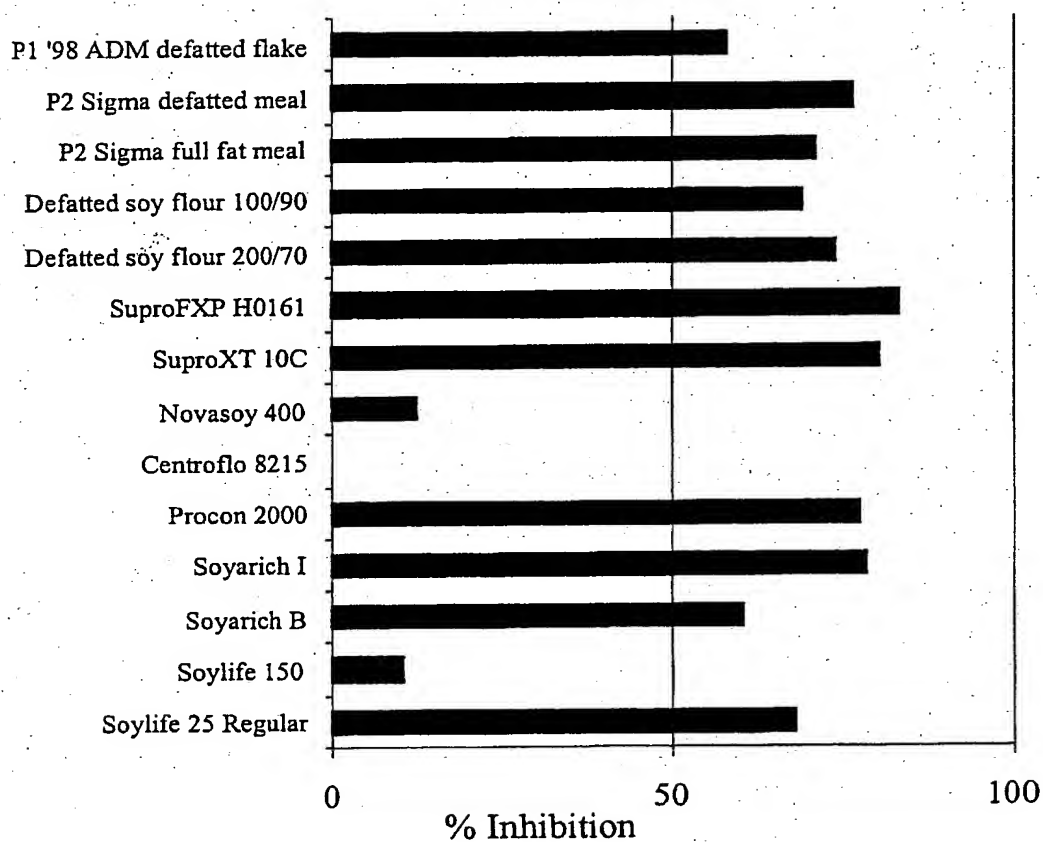


Figure 6A

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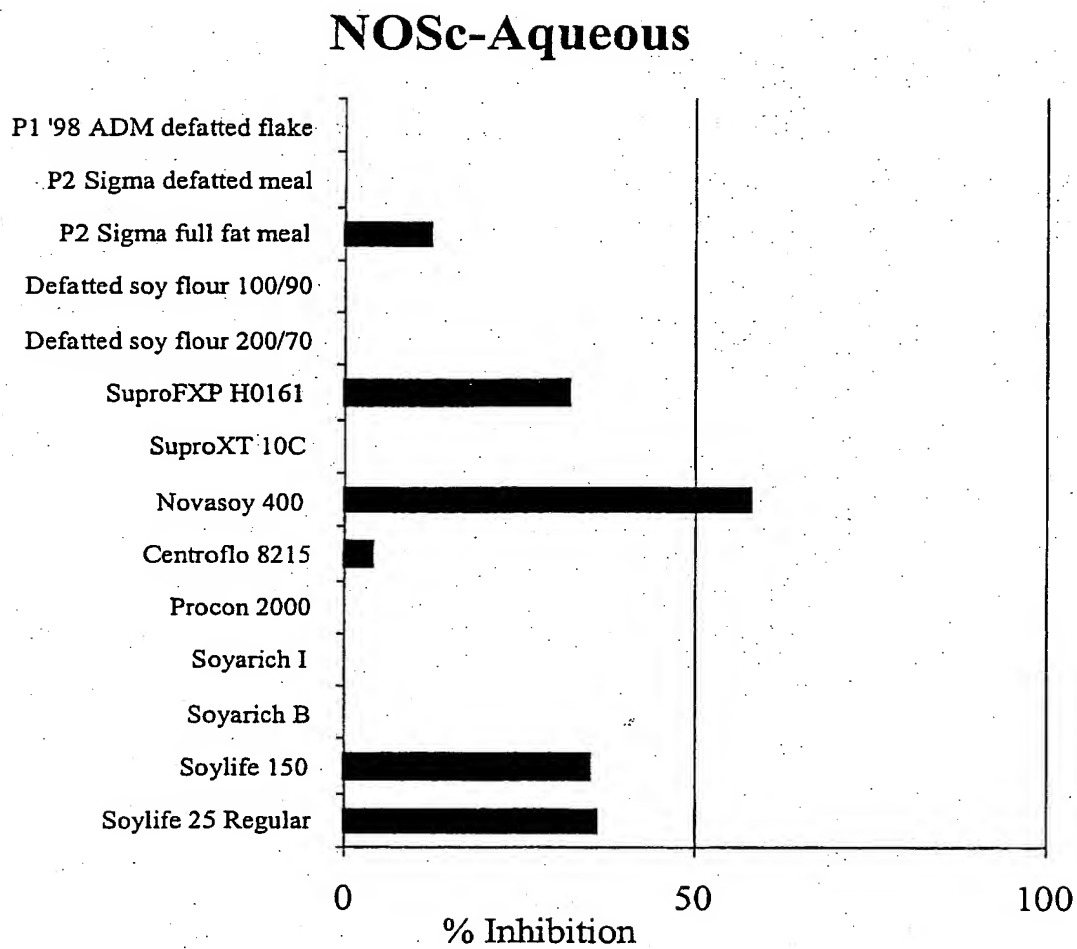


Figure 6B

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## PAF Binding-DMSO

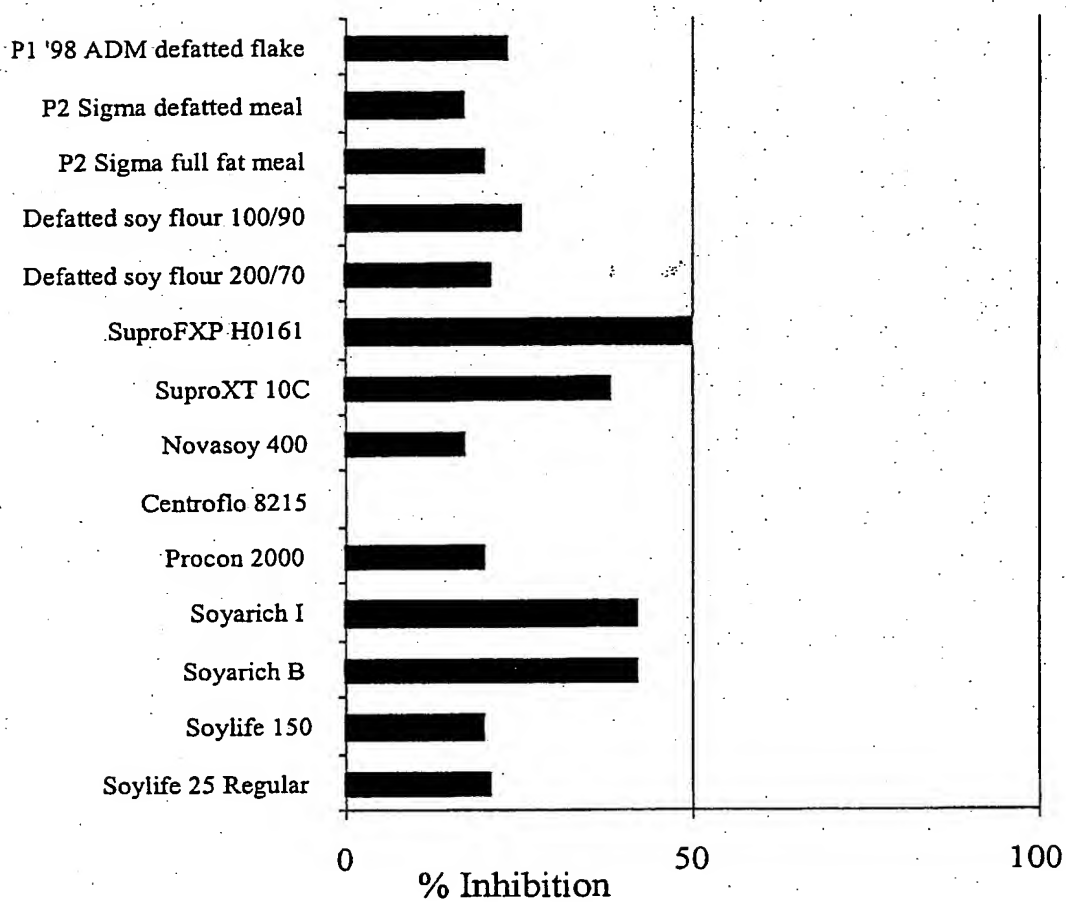


Figure 6C

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### PAF Binding-Ethanol

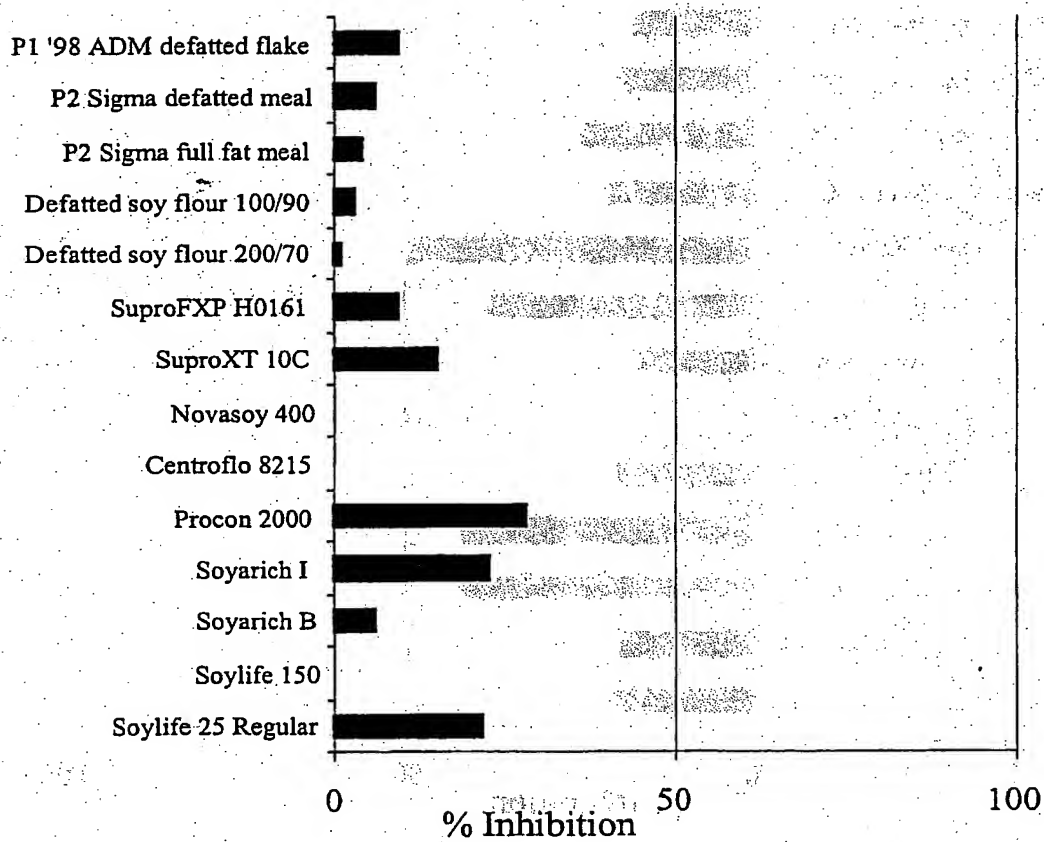


Figure 6D

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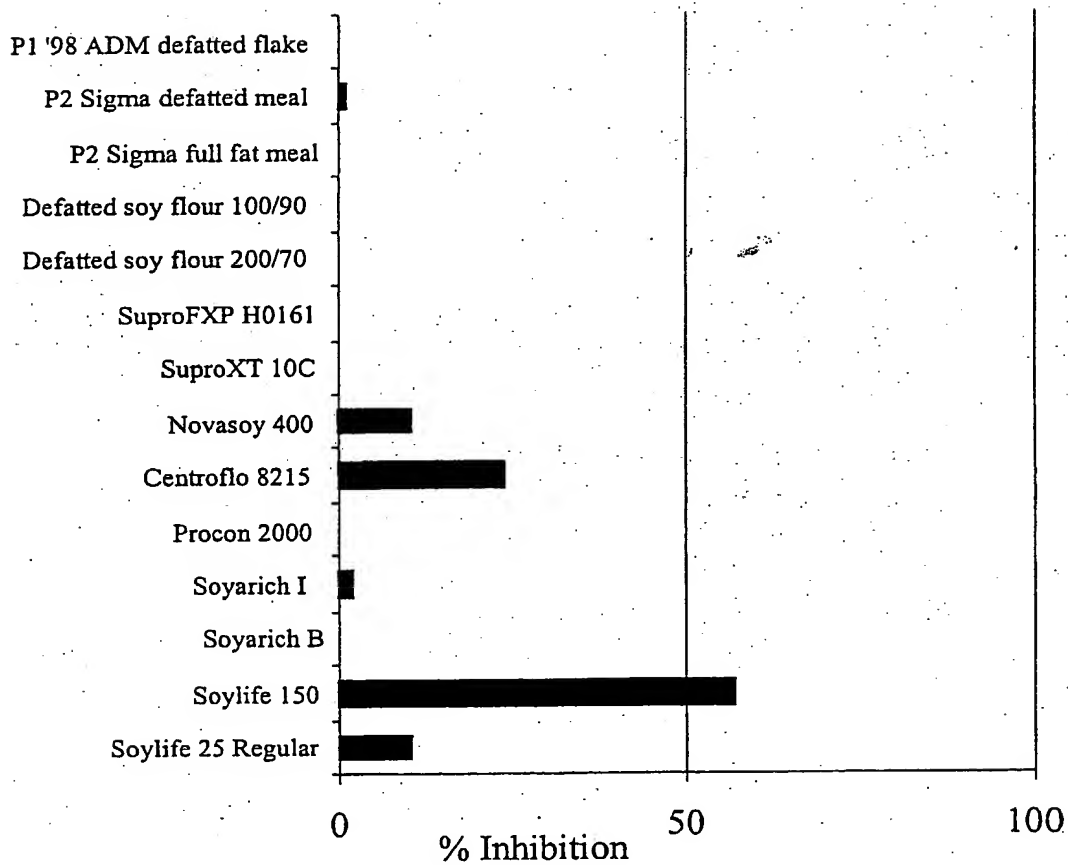
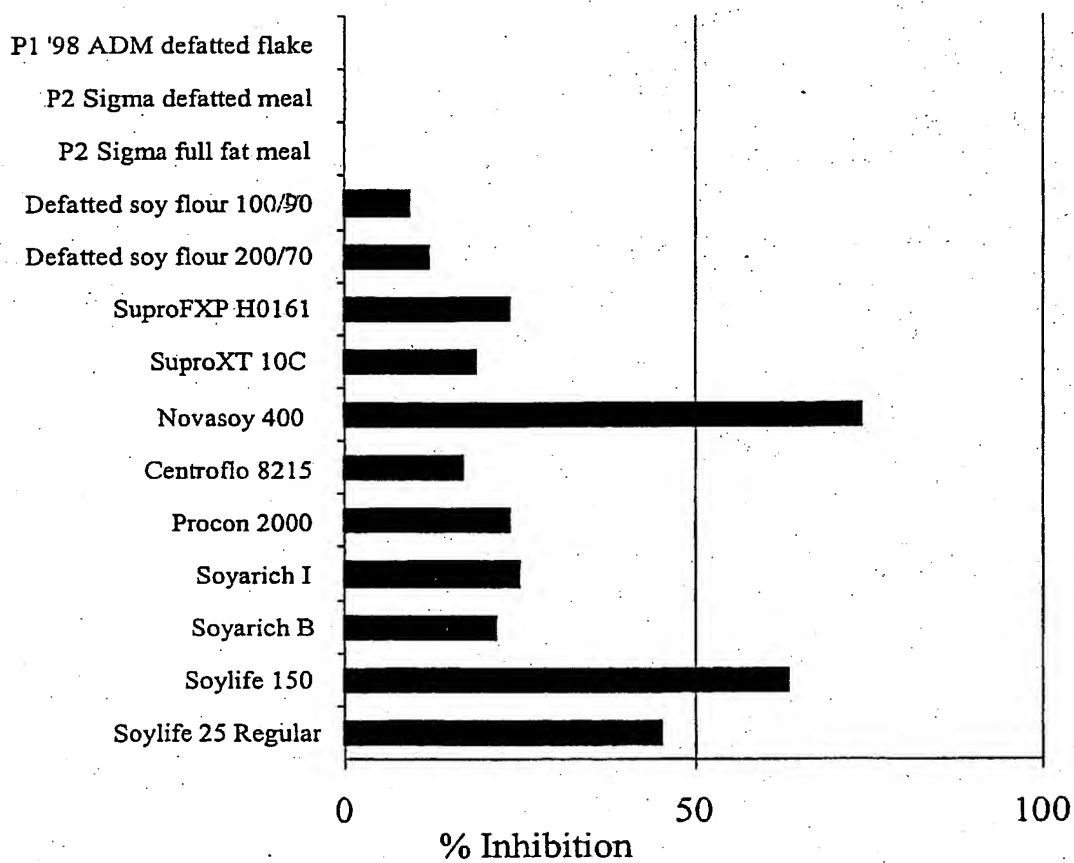
**NOSc-Ethanol**

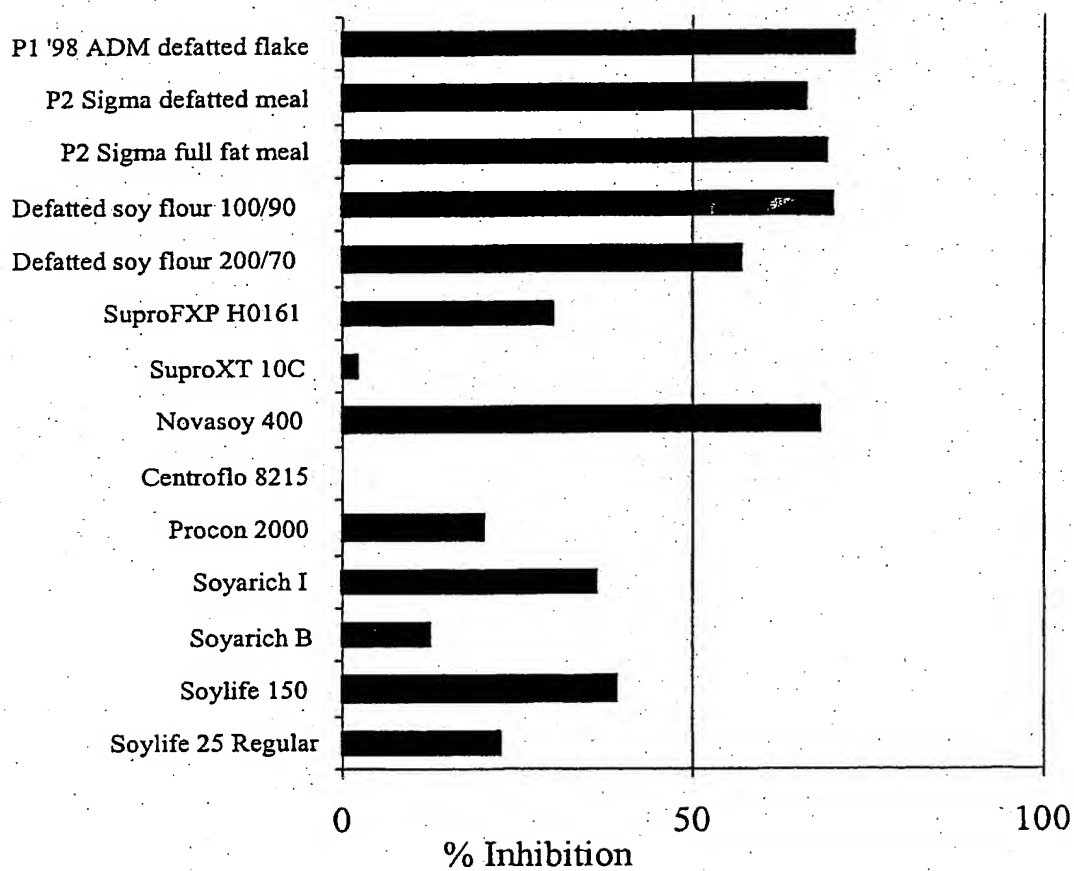
Figure 6E

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**Thromboxane A<sub>2</sub> -Aqueous****Figure 7A**



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**SOD-Aqueous****Figure 7B**

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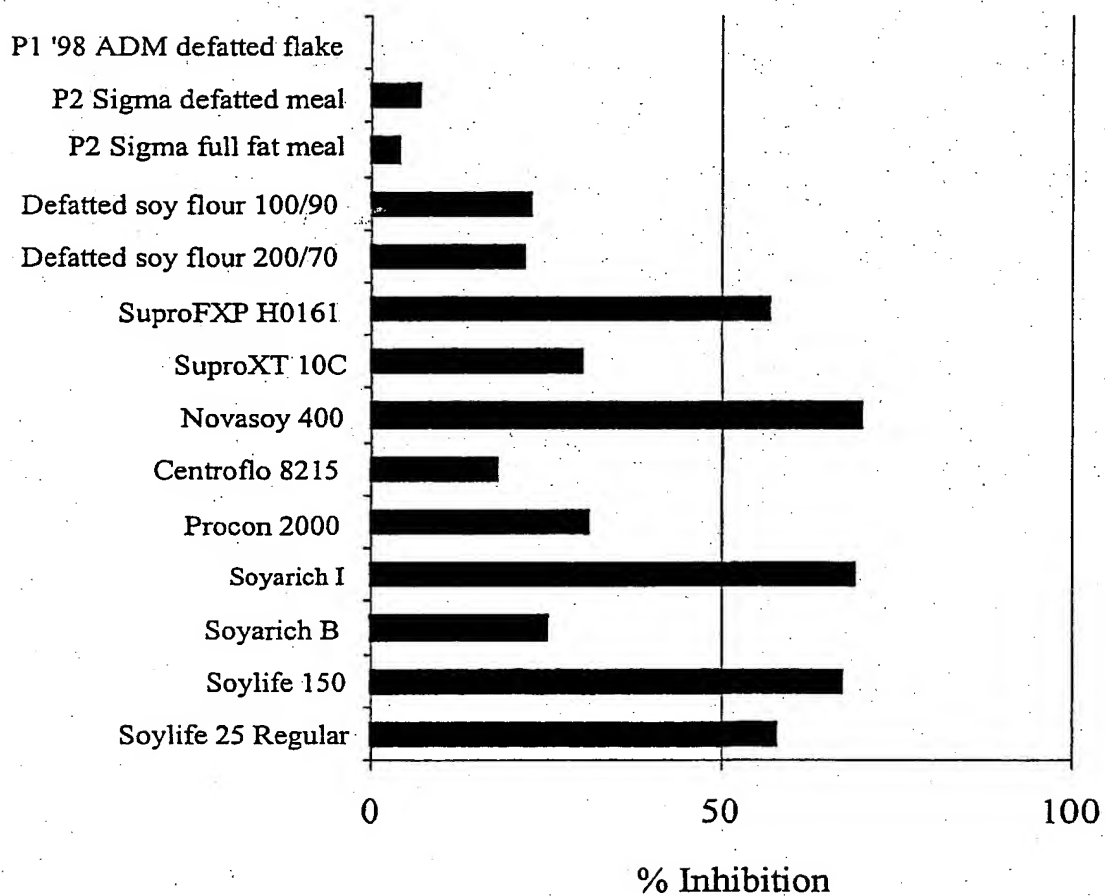
**Thromboxane A<sub>2</sub> -DMSO**

Figure 7C

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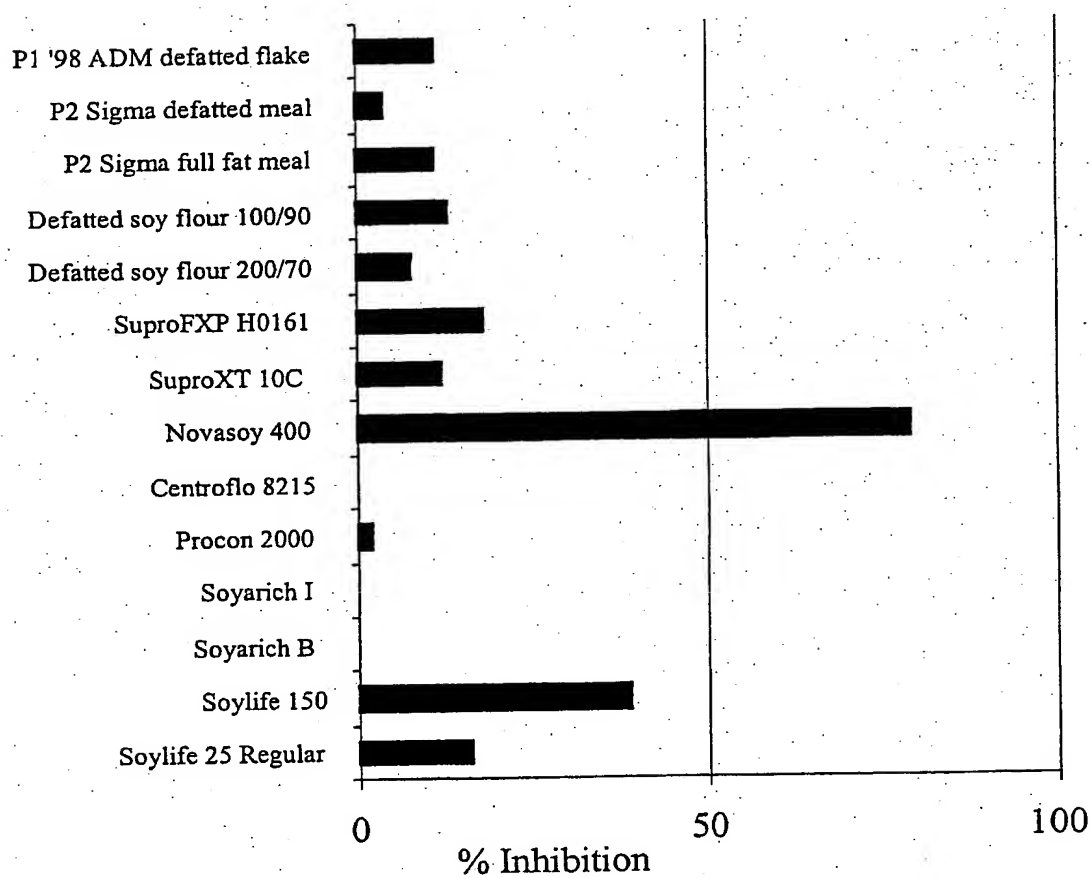
**SOD-DMSO**

Figure 7D

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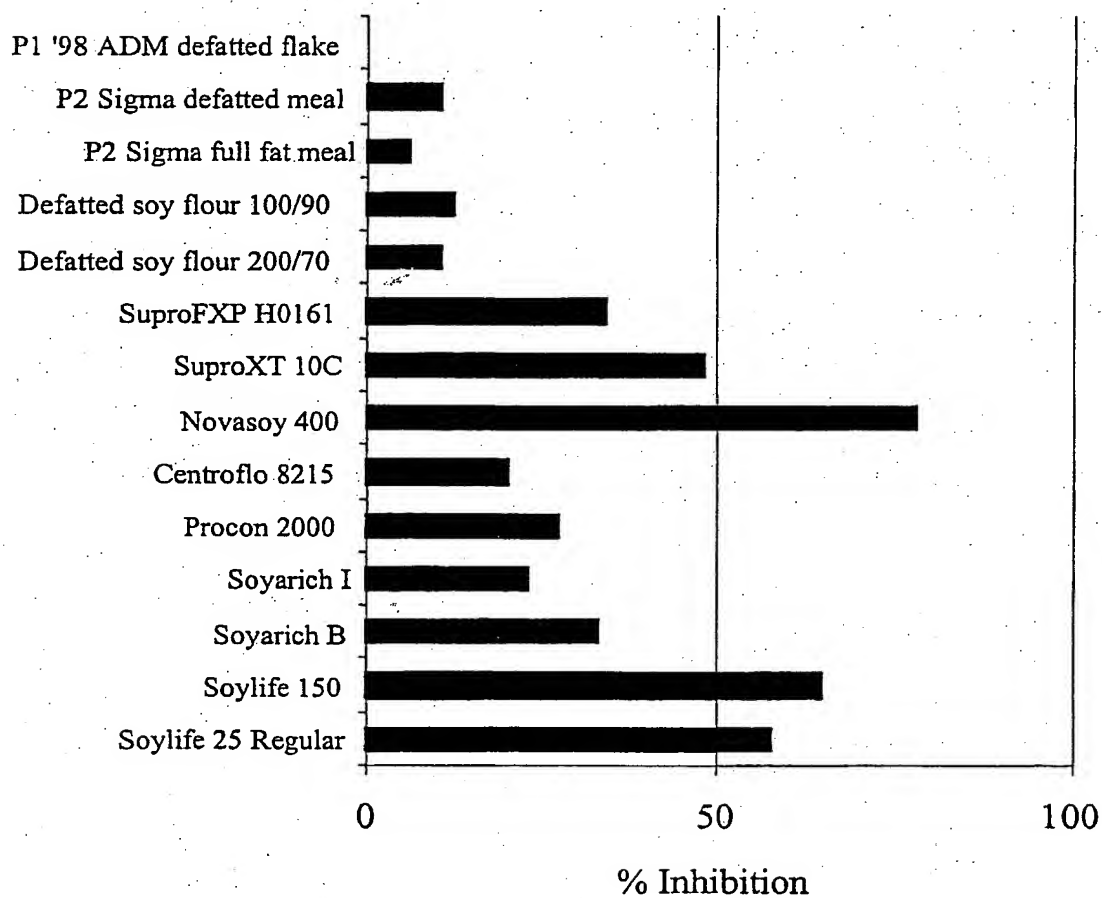
**Thromboxane A<sub>2</sub> -Ethanol**

Figure 7E

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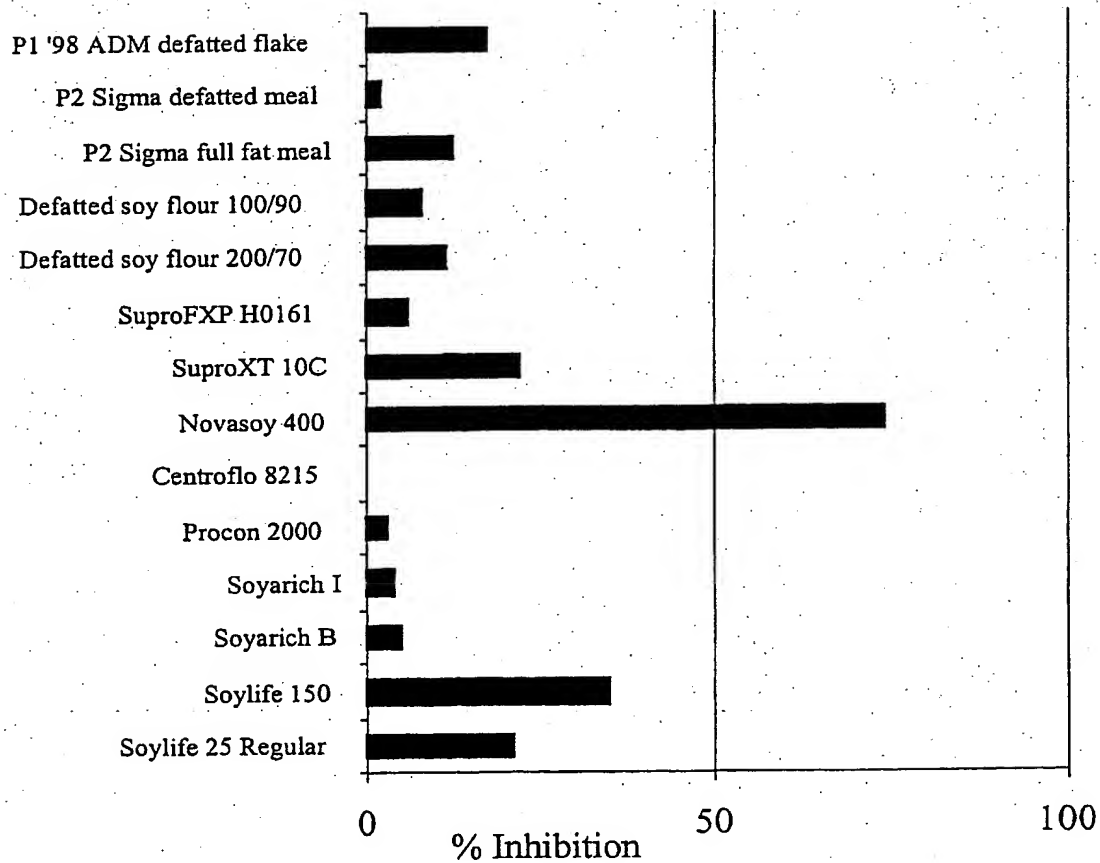
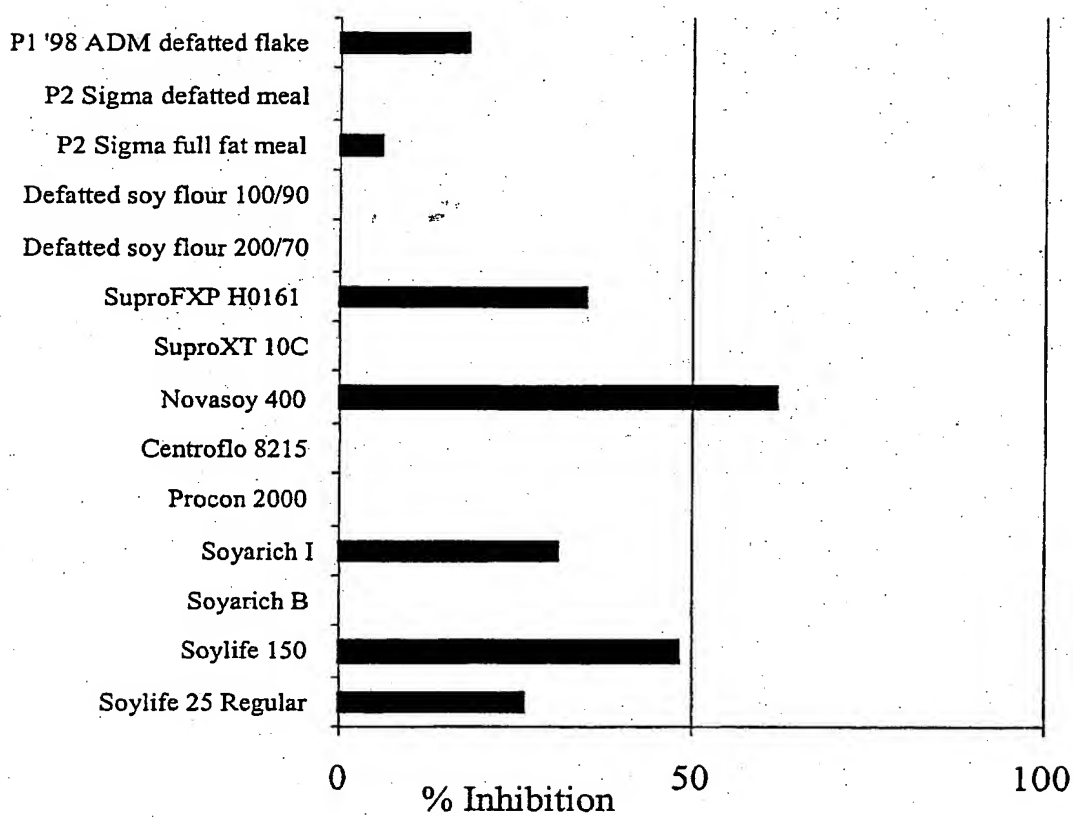
**SOD-Ethanol**

Figure 7F

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**Adrenergic  $\alpha$  2-Aqueous****Figure 8A**

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## Adrenergic $\alpha$ 2-DMSO

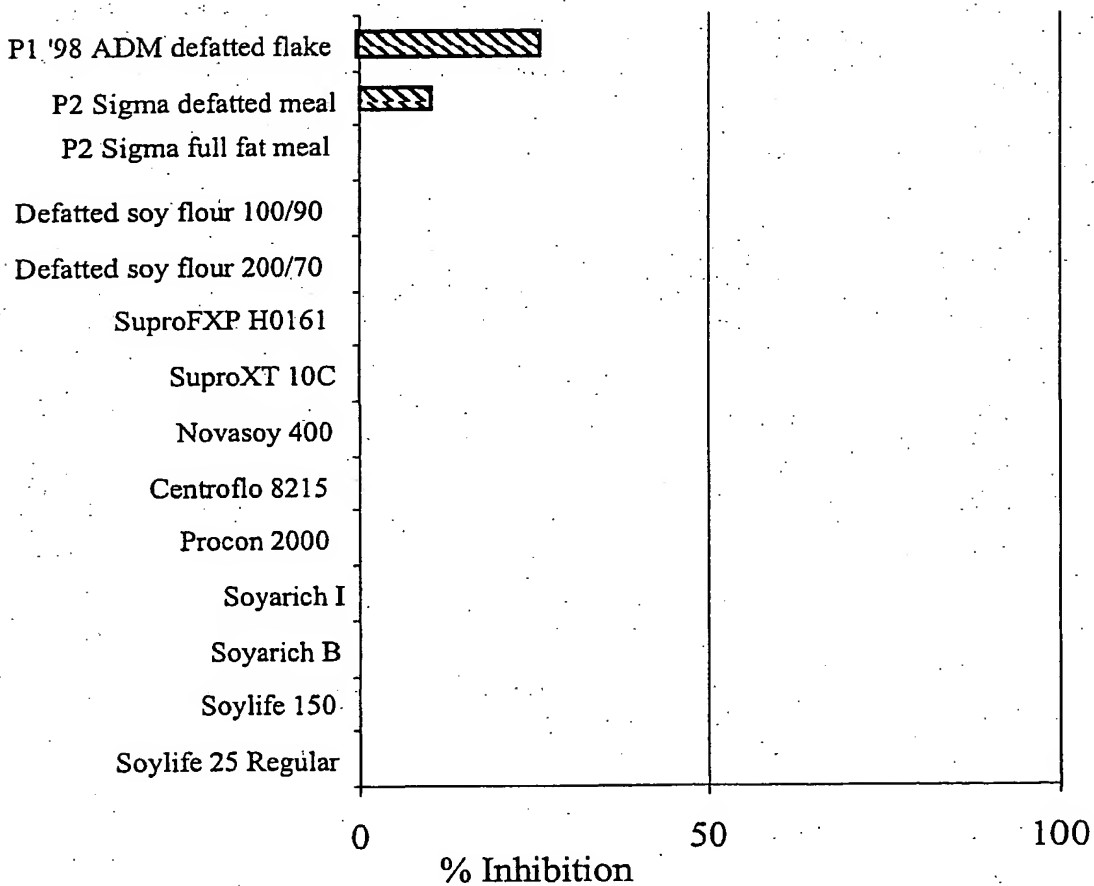
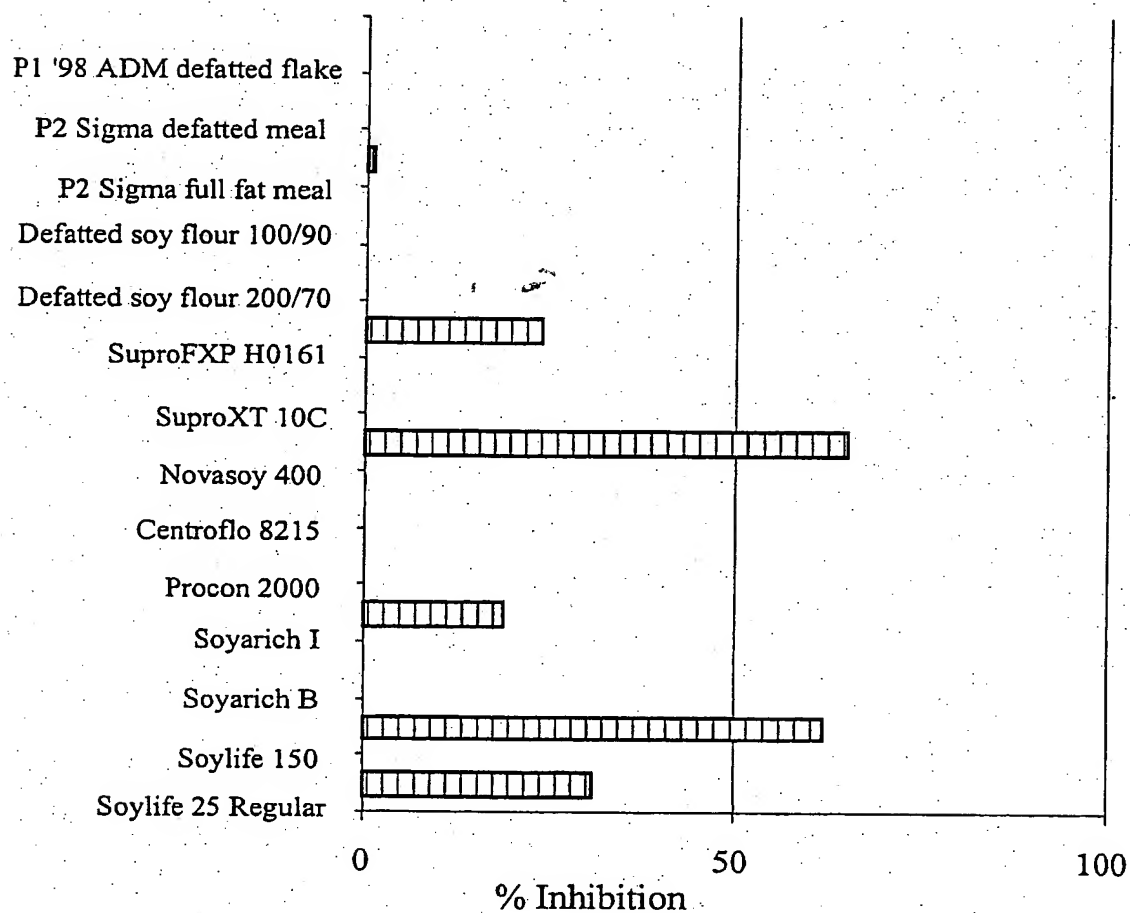


Figure 8B

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**Adrenergic  $\alpha$  2-Ethanol****Figure 8C**



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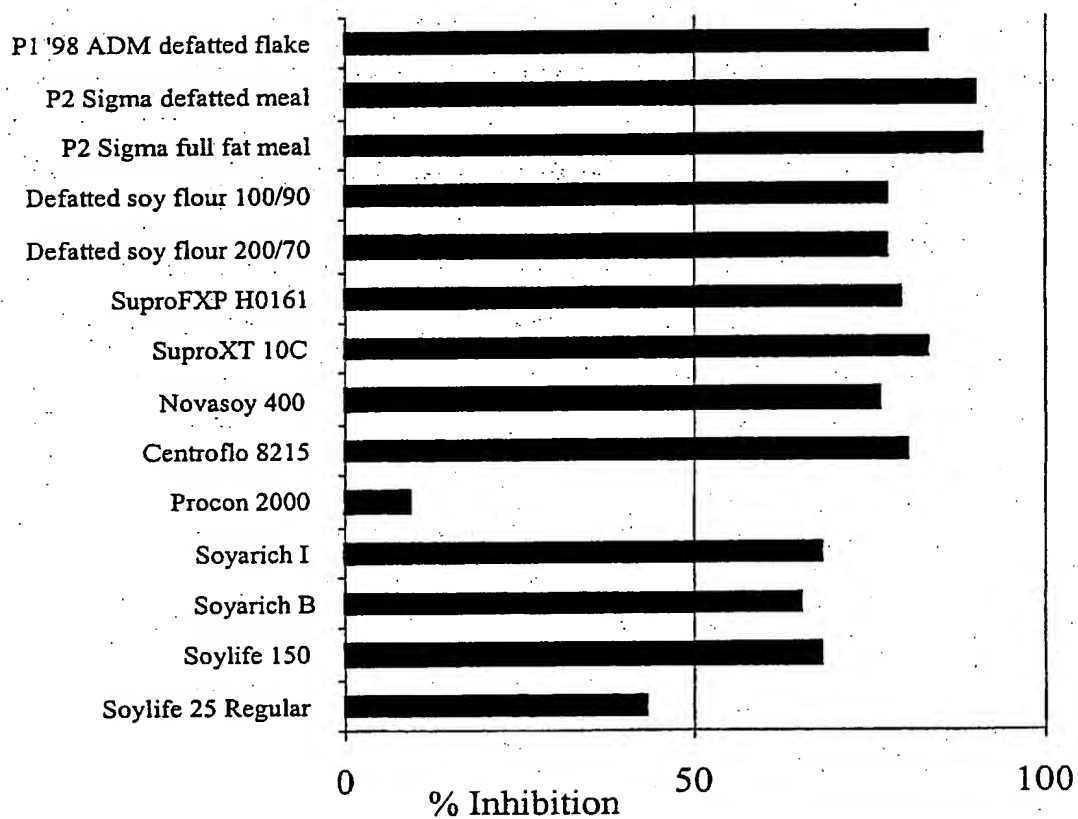
**HER2 TK-Aqueous**

Figure 9A

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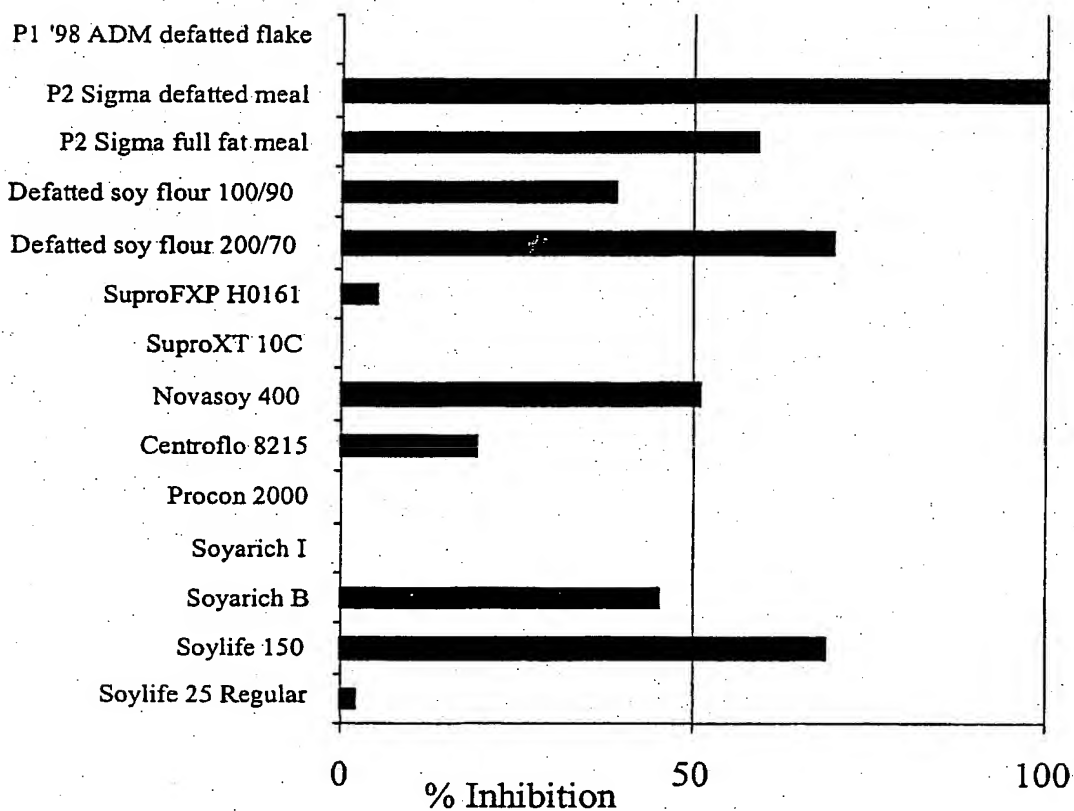
**MAP Kinase-Aqueous**

Figure 9B

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## HER2 TK-DMSO

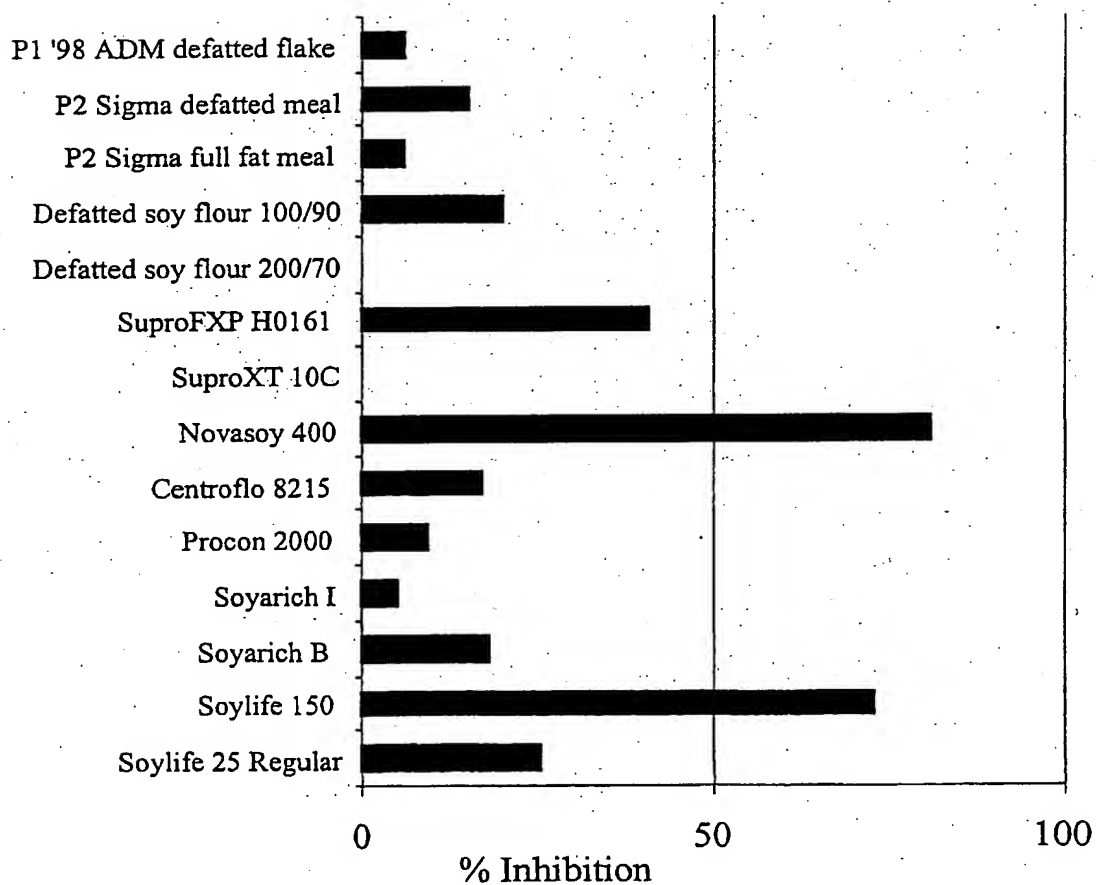


Figure 9C

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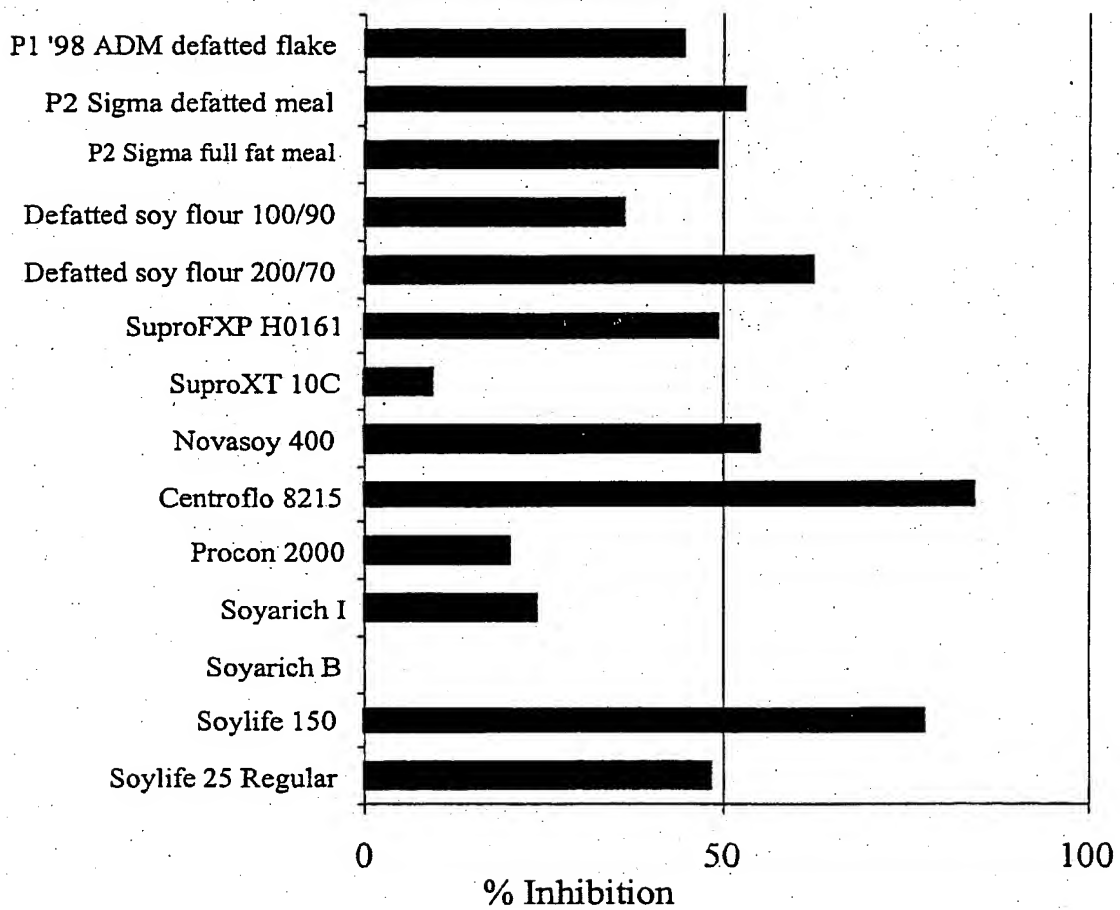
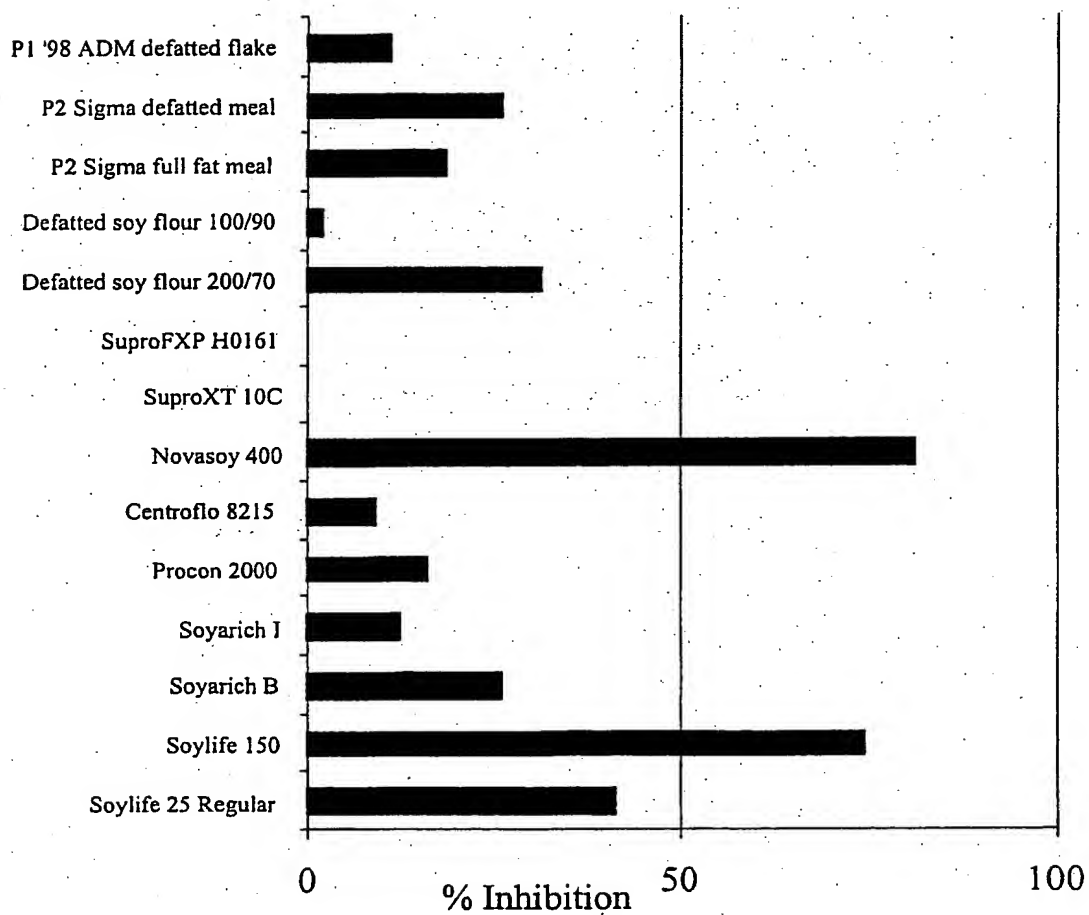
**MAP Kinase-DMSO**

Figure 9D

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**HER2 TK-Ethanol****Figure 9E**

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## MAP Kinase-Ethanol

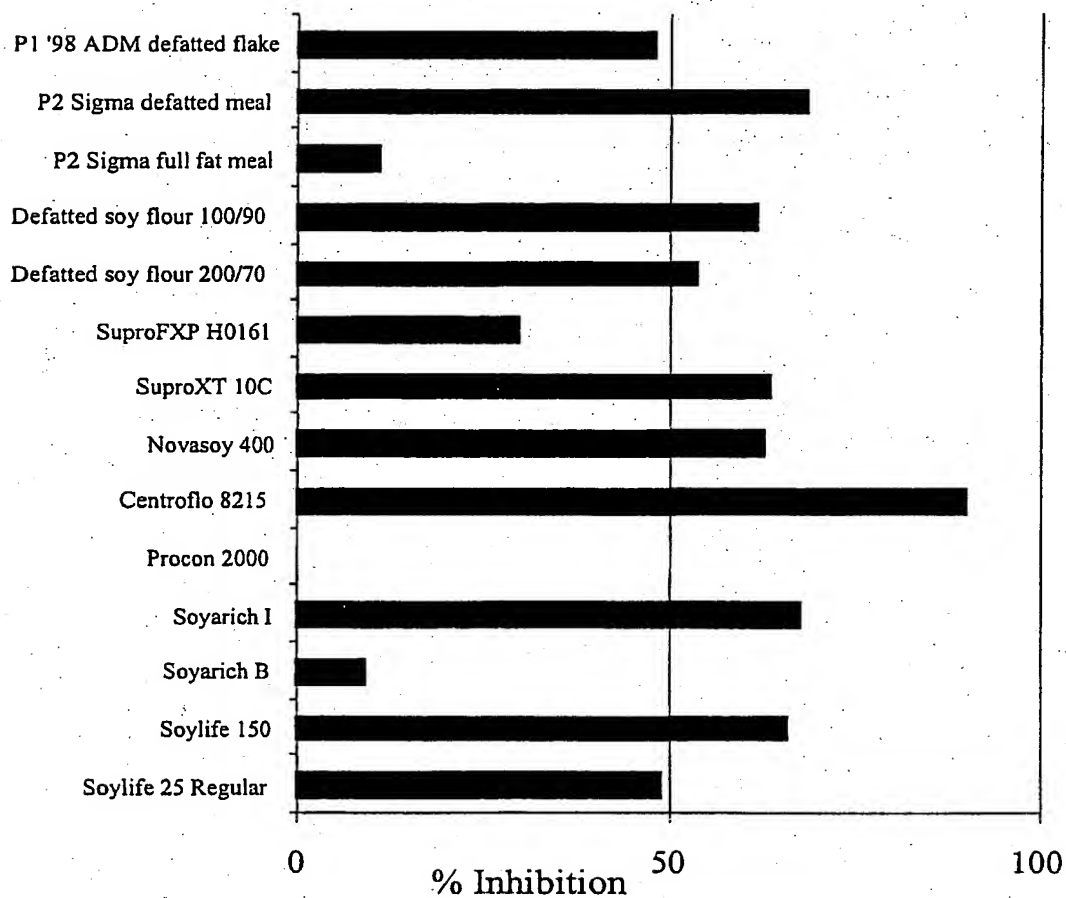


Figure 9F

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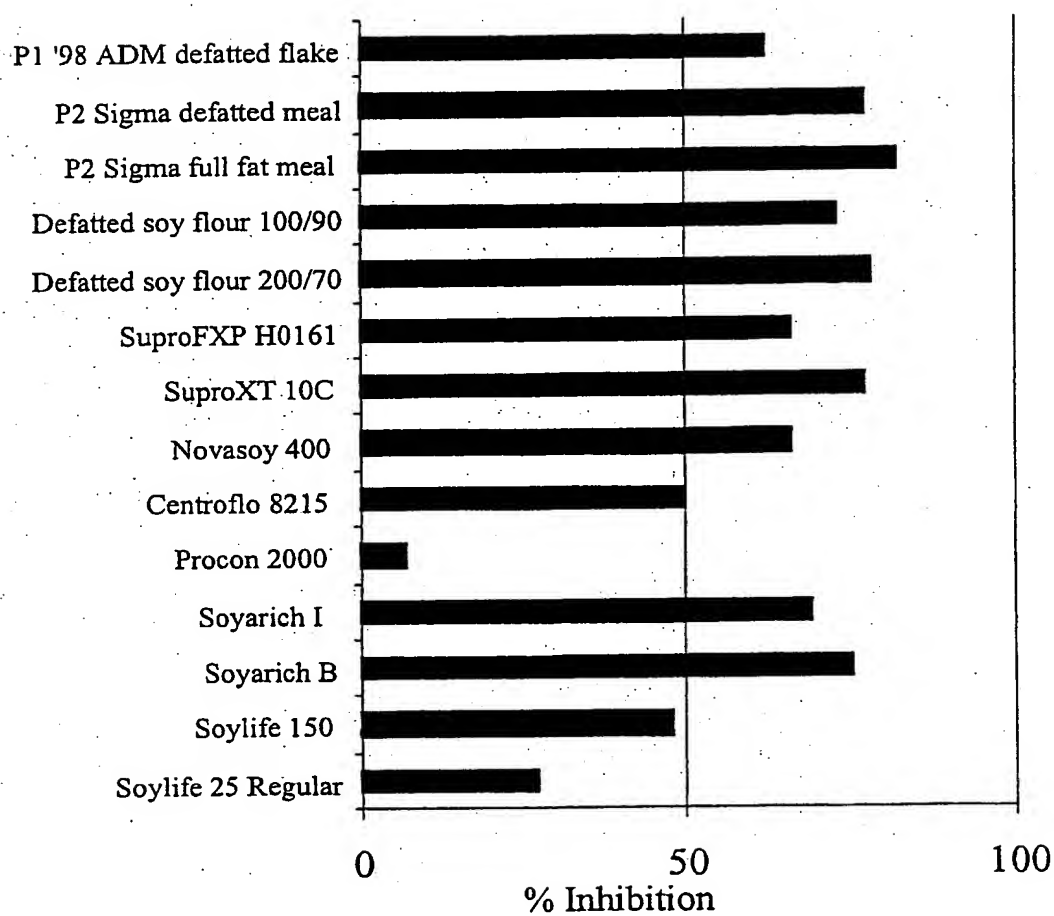
**EGF-R-Aqueous**

Figure 10A

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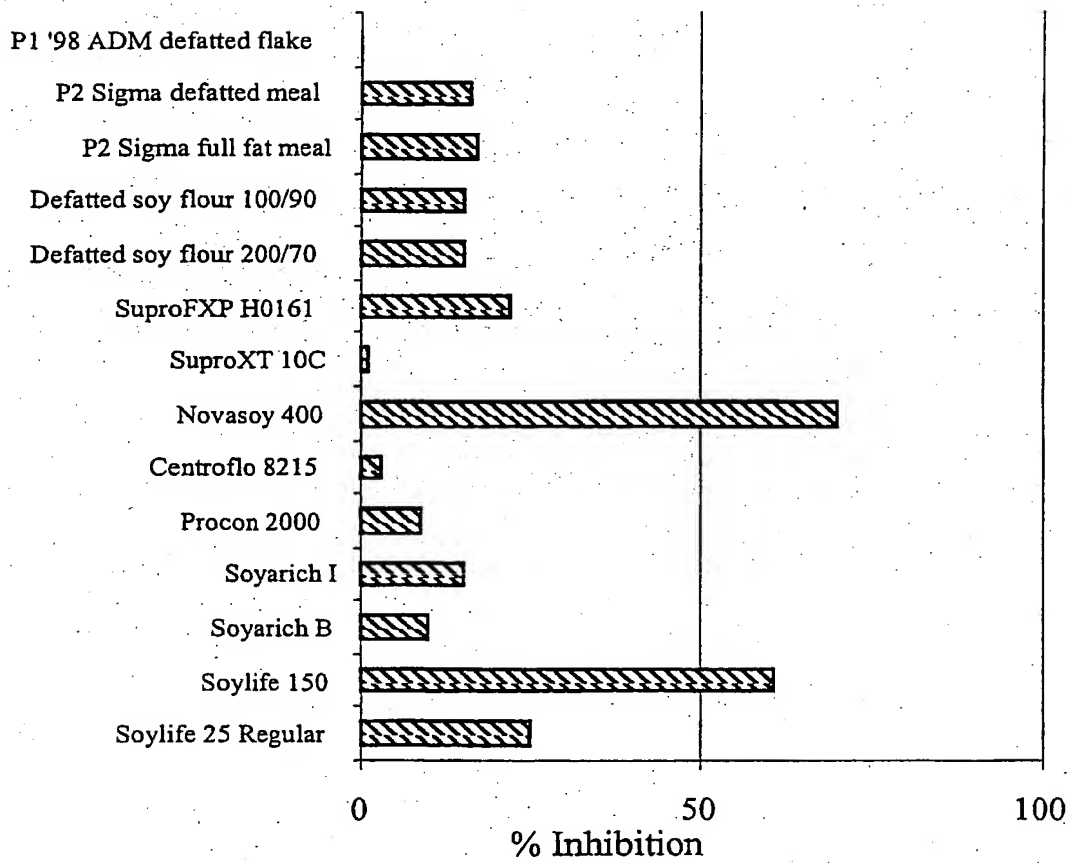
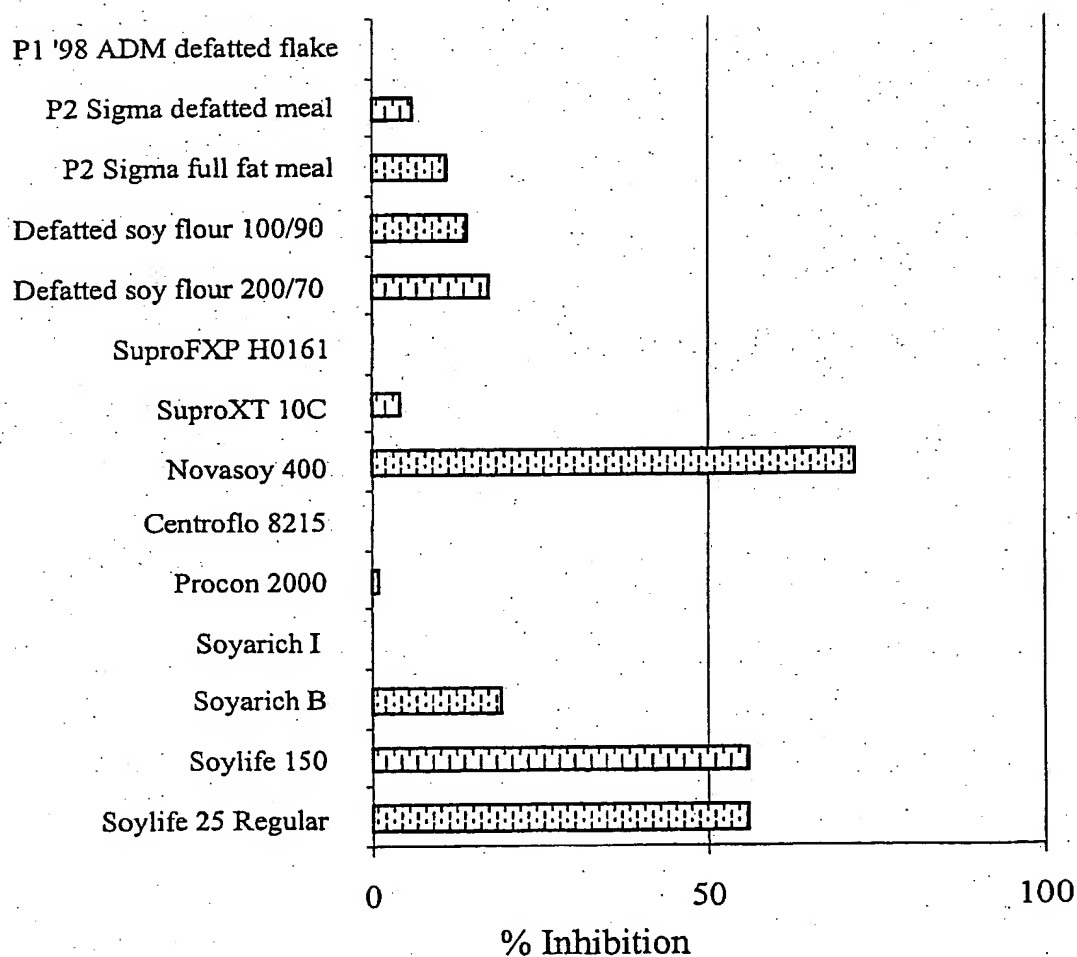
**EGF-R-DMSO**

Figure 10B



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**EGF-R-Ethanol****Figure 10C**

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## Oxytocin-Aqueous

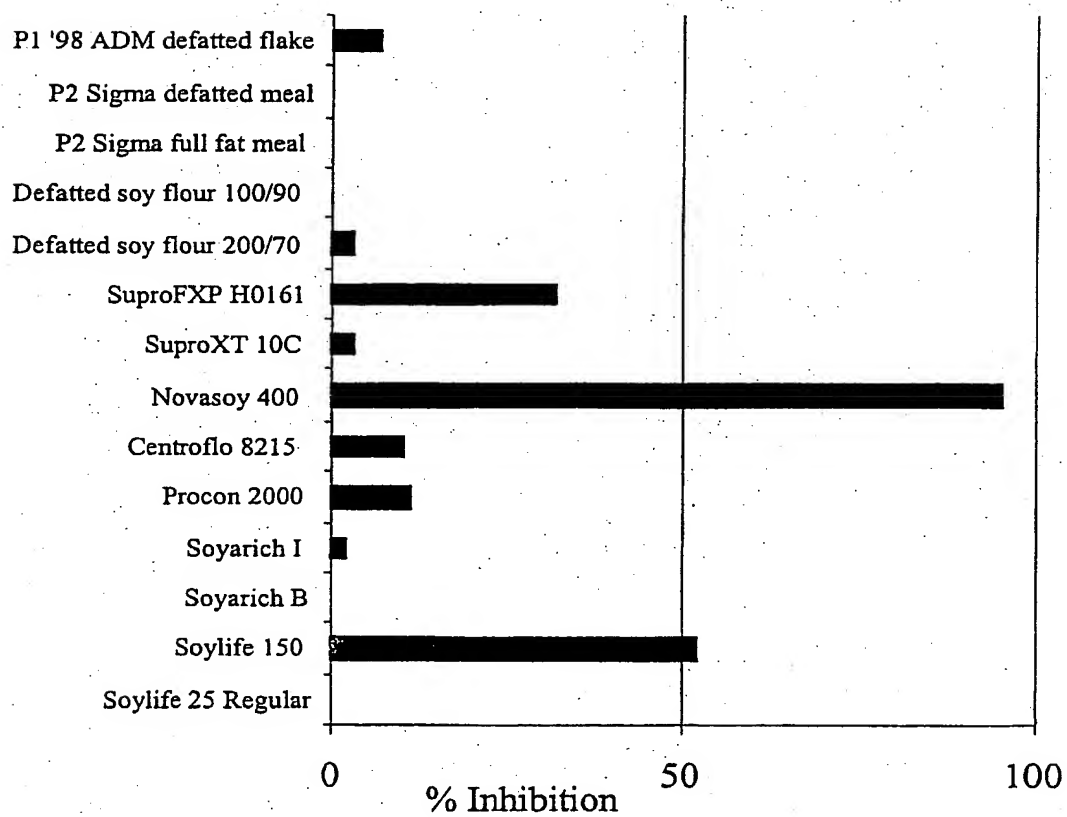


Figure 11A

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### Progesterone-Aqueous

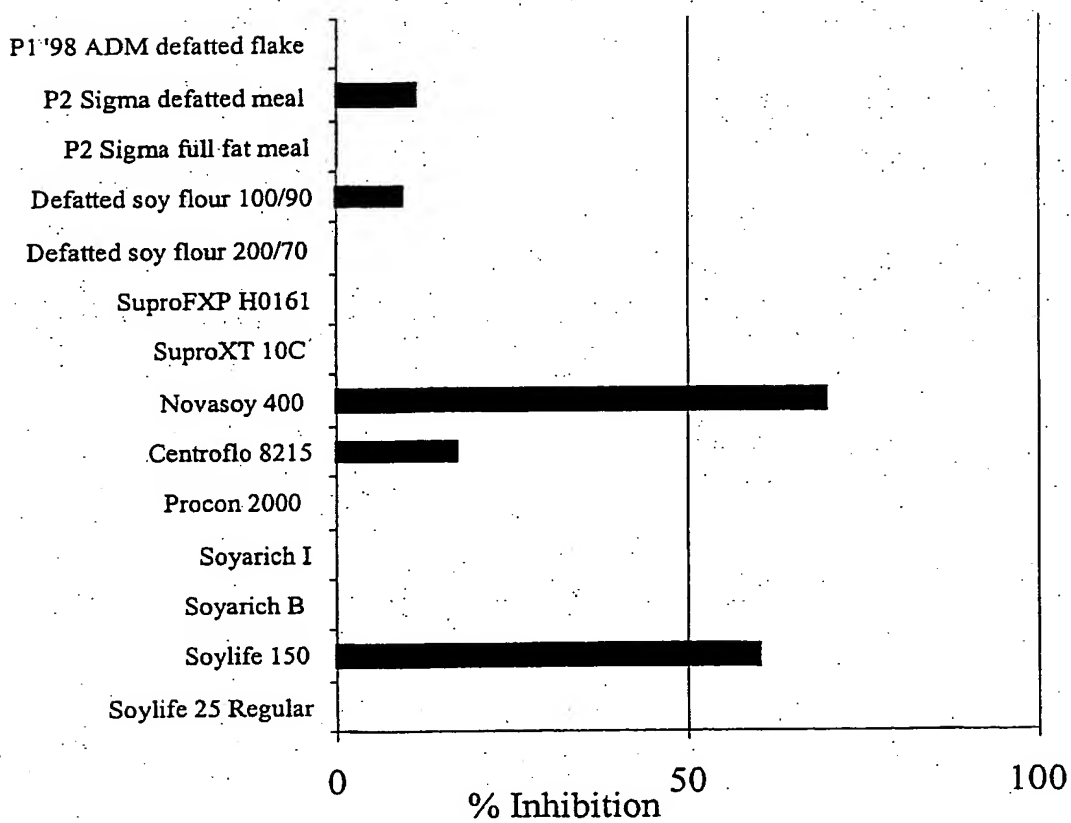


Figure 11B

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## Oxytocin-DMSO

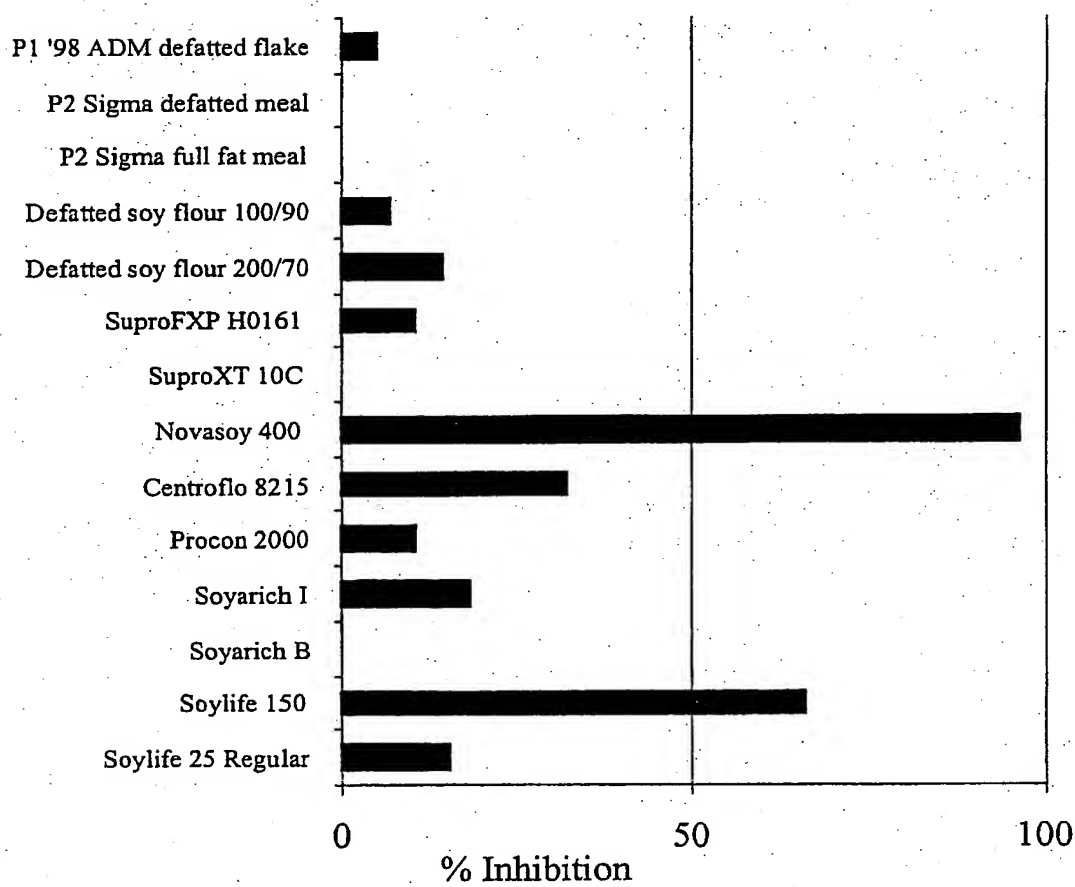


Figure 11C

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## Progesterone-DMSO

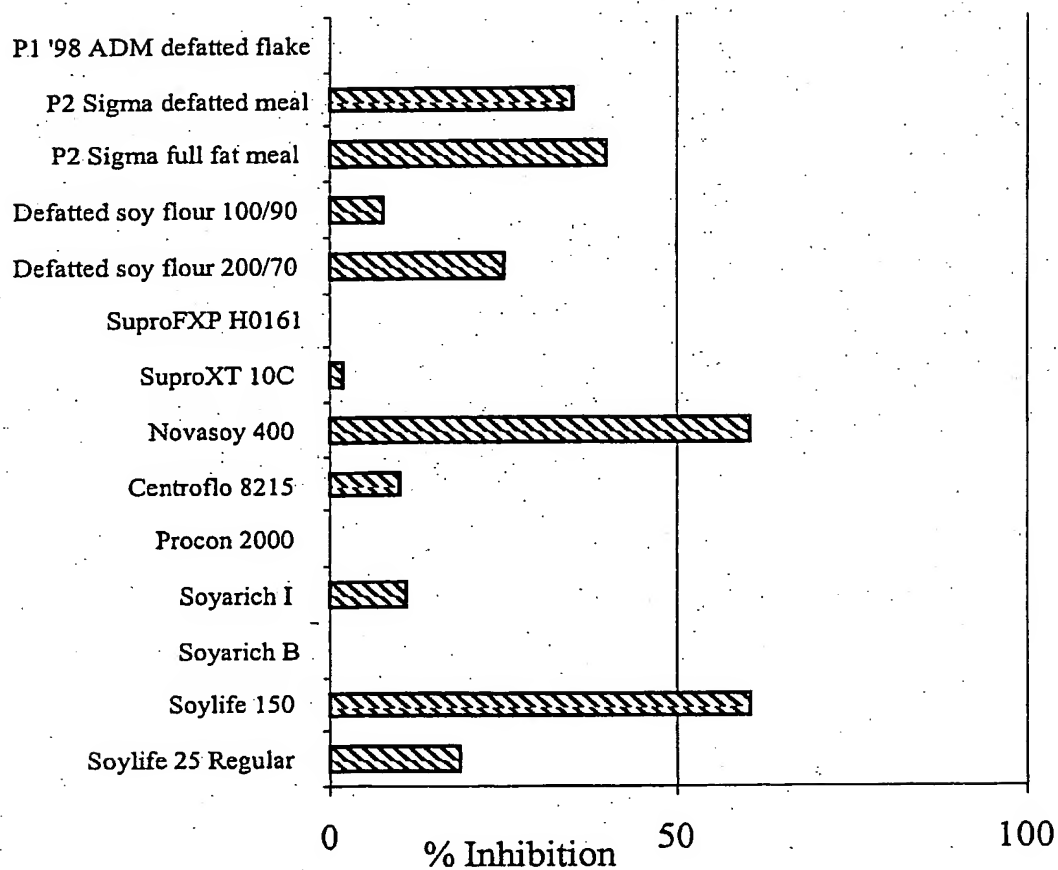
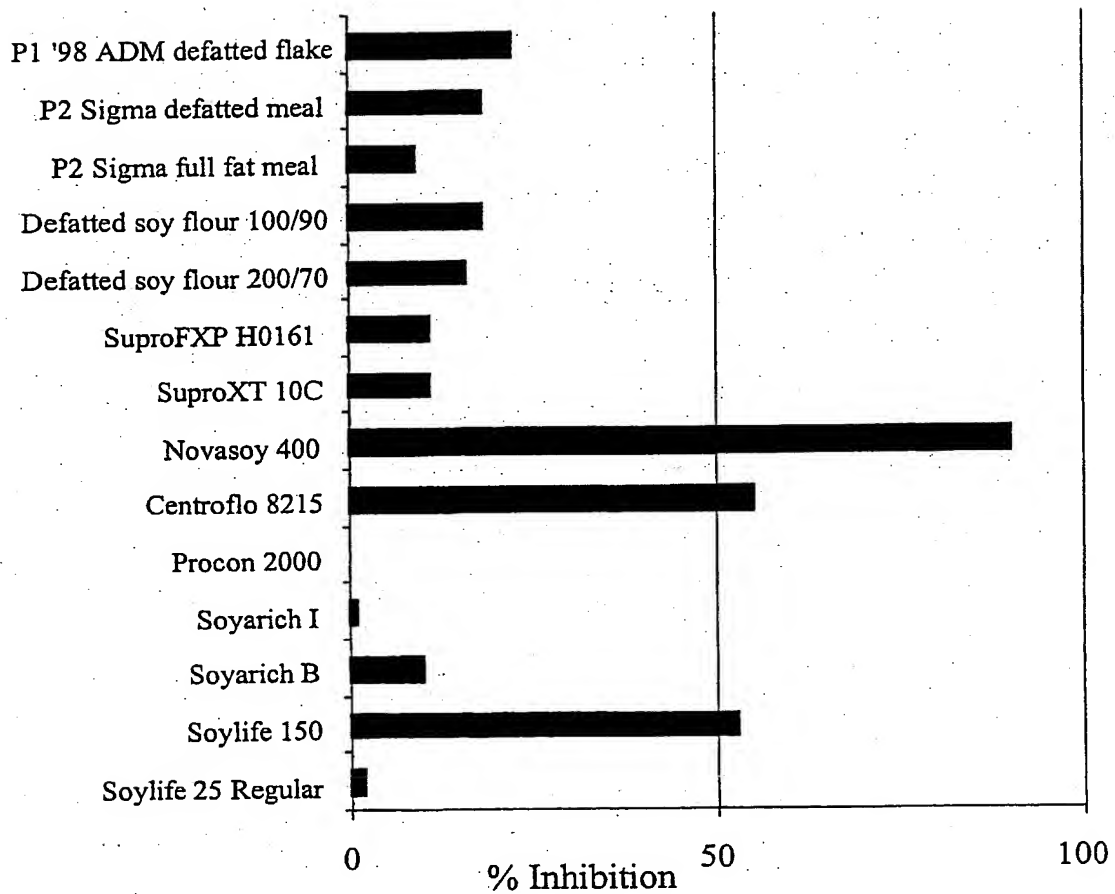


Figure 11D

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## Oxytocin-Ethanol



### Figure 11E

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## Progesterone-Ethanol

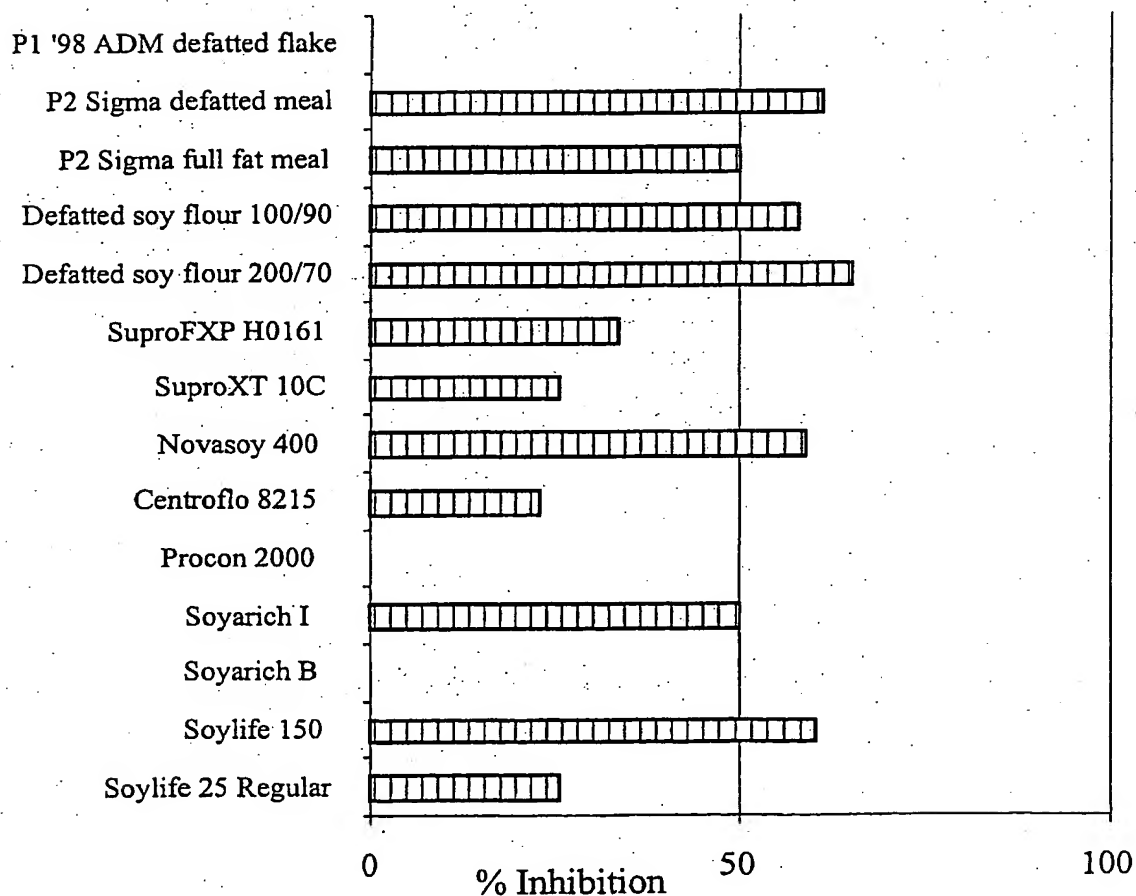


Figure 11F

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## D2 Receptor-Aqueous

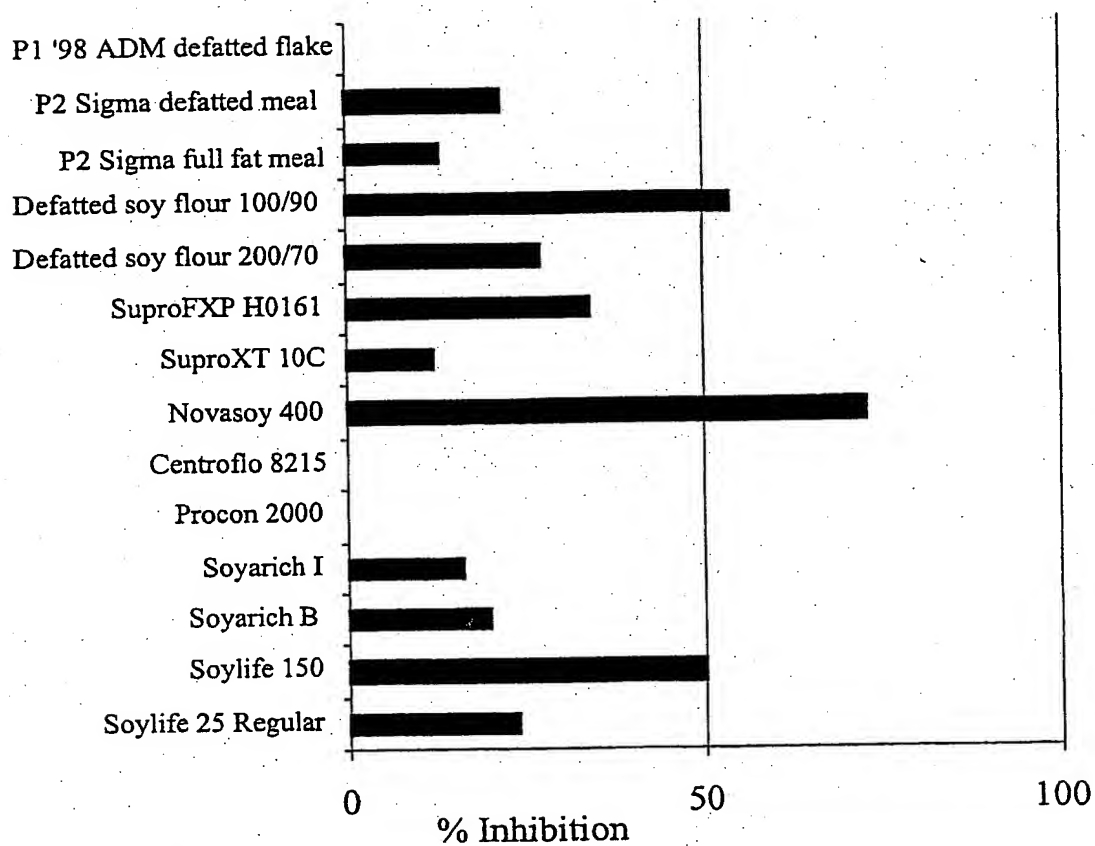


Figure 12A



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## Thyrotropin Releasing Hor.-Aqueous

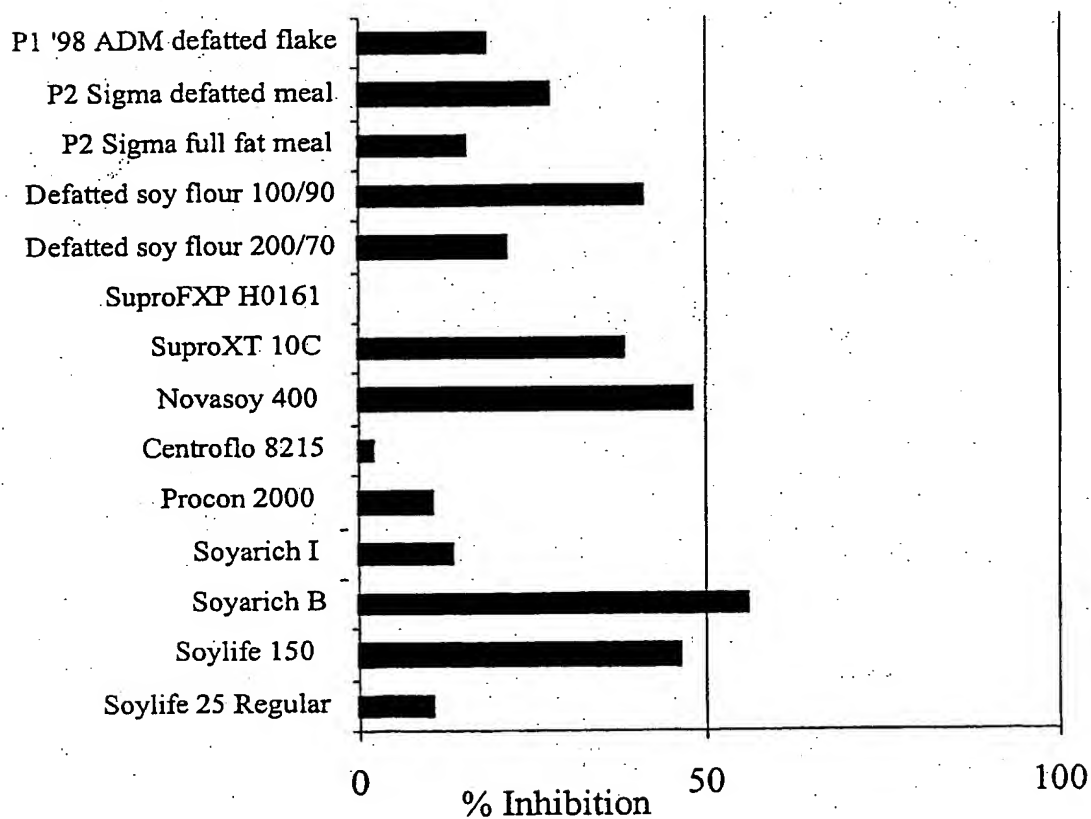


Figure 12B

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## D2 Receptor-Ethanol

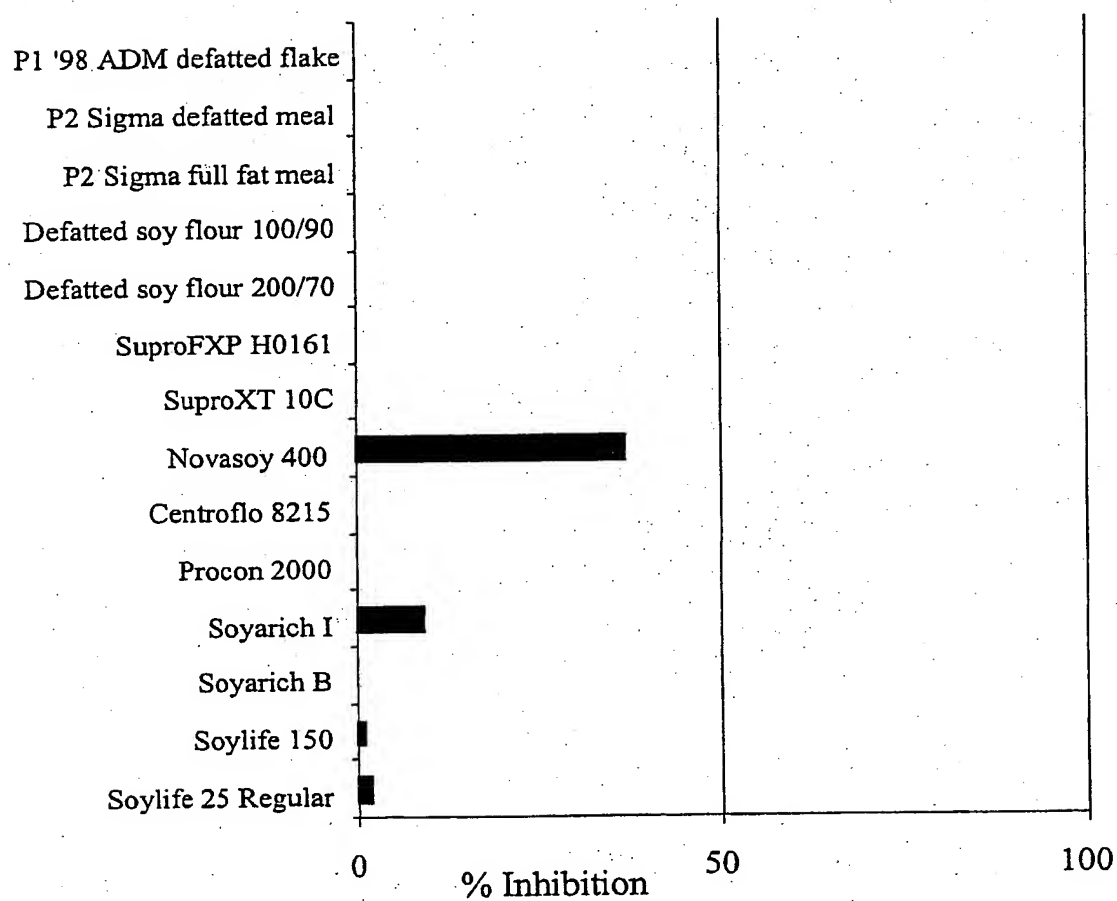


Figure 12C

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## Thyrotropin Releasing Hor.-Ethanol

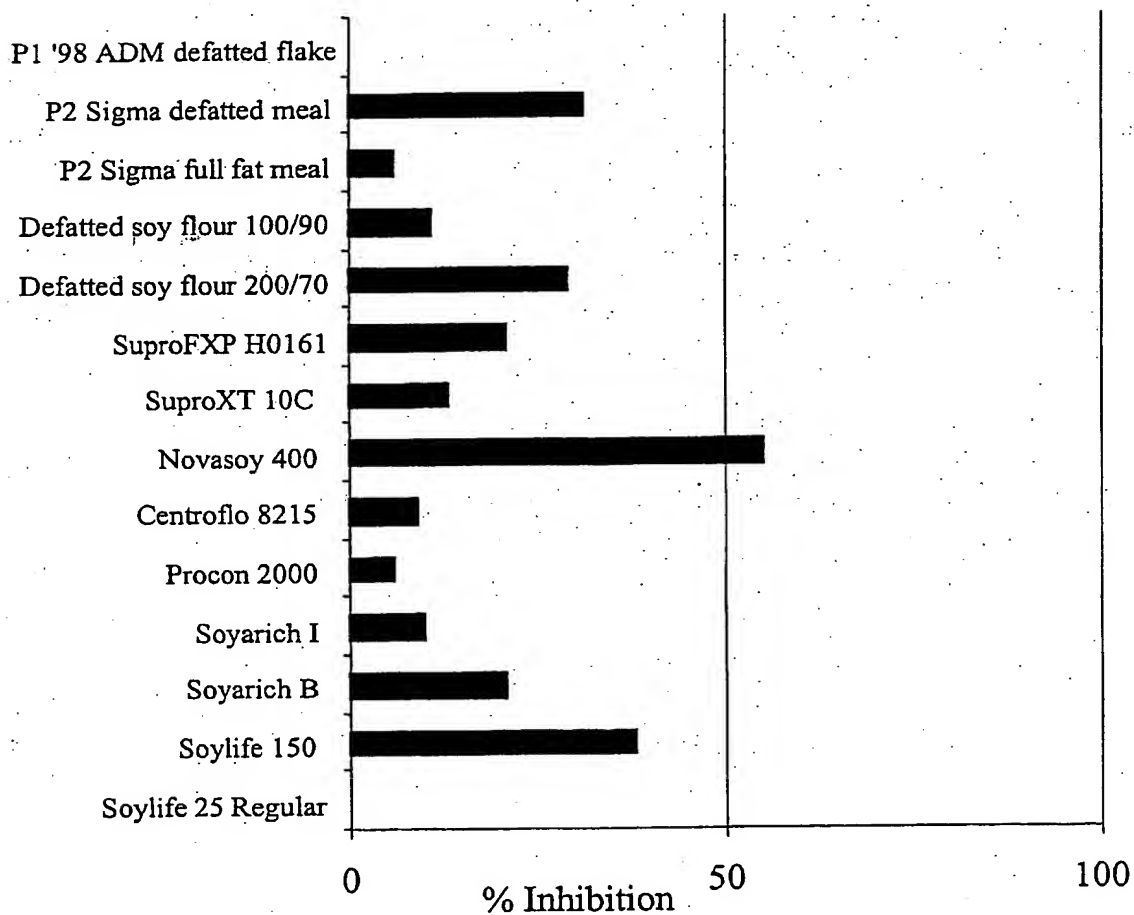


Figure 12D

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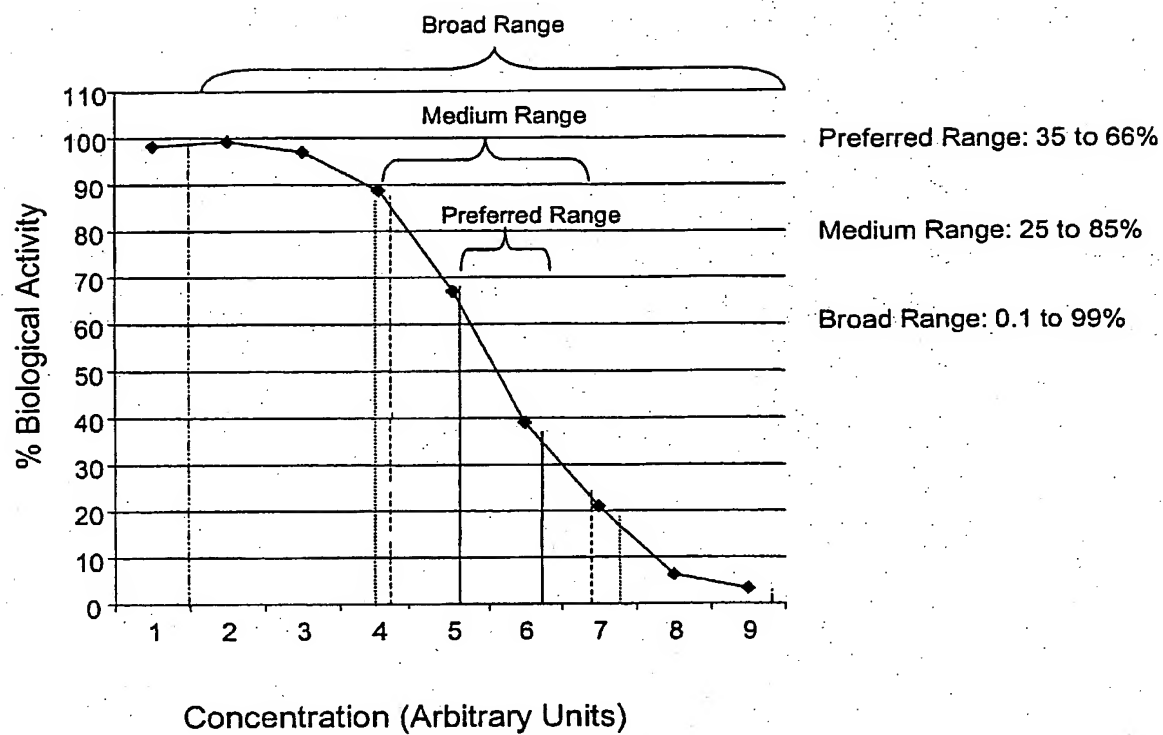


Figure 13